AUGUST 11, 1941

THE JOURNAL OF NUTRITION

VOLUME 22 NUMBER 2

GEORGE R. COWGILL, *Editor*
Yale University School of Medicine, New Haven, Conn.

HAROLD H. MITCHELL, *Associate Editor*
University of Illinois, Urbana, Ill.

EDITORIAL BOARD

ALBERT G. HOGAN — ICIE MACY-HOOBLER — HOWARD B. LEWIS
ERNEST B. FORBES — HENRY A. MATTILL — ALFRED T. SHOHL
PHILIP A. SHAFFER — LELA E. BOOHER — GEORGE O. BURR
CONRAD A. ELVEHJEM — RUSSELL M. WILDER

Official Organ of the American Institute of Nutrition
PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PA.

Price, $5.00 per volume, Domestic; $5.50 per volume, Foreign

Entered as second-class matter January 20, 1934, at the post office at Philadelphia, Pa., under Act of March 3, 1879. Copyright 1941, The Wistar Institute of Anatomy and Biology. All rights reserved.

Publications of The Wistar Institute

JOURNAL OF MORPHOLOGY

Devoted to the publication of original research on animal morphology and physiology, including cytology, protozoology, and the embryology of vertebrates and invertebrates. Articles do not usually exceed 50 pages in length.

Issued bimonthly, 2 vols. annually: $20.00 Domestic, $21.00 Foreign, per year.

THE JOURNAL OF COMPARATIVE NEUROLOGY

Publishes the result of original investigations on the comparative anatomy and physiology of the nervous system.

Issued bimonthly, 2 vols. annually: $15.00 Domestic, $16.00 Foreign, per year.

THE AMERICAN JOURNAL OF ANATOMY

Devoted primarily to the interests of anatomy as taught in the medical schools of the United States and to the development of scientific anatomy in these schools. Publishes researches only.

Issued bimonthly, 2 vols. annually: $15.00 Domestic, $16.00 Foreign, per year.

THE ANATOMICAL RECORD

Organ of the American Association of Anatomists and the American Society of Zoologists

For the prompt publication of concise original articles on vertebrate anatomy, preliminary reports; technical notes; critical notes of interest to anatomists and short reviews of noteworthy publications.

Issued monthly, 3 vols. annually: $22.50 Domestic, $24.00 Foreign, per year.

THE JOURNAL OF EXPERIMENTAL ZOOLOGY

Publishes papers embodying the results of original researches of an experimental or analytical nature in the field of zoology.

Issued 9 times a year, 3 vols. annually: $22.50 Domestic, $24.00 Foreign, per year.

AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY

Organ of the American Association of Physical Anthropologists

In this journal appear original papers on anthropology in general; researches in evolution, man's origin; human ontogeny; variations in man; heredity; eugenics; demography; the American Indian and abnormal classes. Papers on anthropometry and the methods of anthropology also appear.

Issued quarterly, 1 vol. annually: $6.00 Domestic, $6.50 Foreign, per year.

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

Publishes papers which embody the results of original research of a quantitative or analytical nature in general and comparative physiology, including both their physical and chemical aspects.

Issued bimonthly, 2 vols. annually: $10.00 Domestic, $11.00 Foreign, per year.

THE JOURNAL OF NUTRITION

Organ of the American Institute of Nutrition

Publishes original researches in the field of nutrition and occasional reviews of literature on topics with which the journal is concerned.

Issued monthly, 2 vols. annually: $10.00 Domestic, $11.00 Foreign, per year.

THE AMERICAN ANATOMICAL MEMOIRS

Publishes original monographs based on experimental or descriptive investigations in the field of anatomy which are too extensive to appear in the current periodicals. Each number contains only one monograph. List of monographs already published, with prices, sent on application.

ADVANCE ABSTRACT CARD SERVICE

Every paper accepted for publication in one of the above periodicals is accompanied by the author's abstract. The abstract and the complete bibliographic reference to the paper as it will eventually appear is printed on the face of a standard library catalogue card. This Advance Abstract Card Service is issued promptly, in advance of the journal containing the paper, and is offered in three styles.

Prices per year $2.00, $2.50 and $3.00, postpaid.

These publications enjoy the largest circulation of any similar journals published.

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

Woodland Avenue and Thirty-sixth Street, Philadelphia, Pa.

THE EXCRETION OF SELENIUM BY RATS ON A SELENIFEROUS WHEAT RATION [1]

H. D. ANDERSON AND A. L. MOXON
South Dakota Agricultural Experiment Station, Brookings

TWO FIGURES

(Received for publication February 14, 1941)

Students of public health frequently raise the question whether animals which have subsisted on seleniferous rations are safe for human consumption. The answer can hardly be forthcoming until more is known of the actual effects of selenium on man. At present, outside of certain skin lesions (Lemley, '40) due to selenium sensitivity, little is known of the human pathology due to the ingestion of selenium as it may occur in foods.

With the above question in mind, it becomes of interest to determine how much selenium is stored by the animal and how fast and by what means this element is excreted.

In this laboratory Moxon ('41) observed that when rats were fed a toxic wheat ration containing 18 p.p.m. of selenium, the fecal excretion of selenium increased from 16% to 22% of the ingested selenium over a period of 6 weeks. The amount excreted in the urine increased from 20.2% to 35.5% of the selenium ingested. Thus the animals retained 63.5% of the selenium intake during the first week. The percentage of retained selenium gradually but steadily decreased until the sixth week at which time it amounted to but 40%. This continual decrease in selenium retention, signifying an approach to an equilibrium state, suggested that a saturation

[1] Approved for publication by the director of the South Dakota Agricultural Experiment Station as journal series 135.

THE JOURNAL OF NUTRITION, VOL. 22, NO. 2
AUGUST, 1941
Copyright 1941
The Wistar Institute of Anatomy and Biology.
All rights reserved.

process was involved. The point of equilibrium or saturation was not reached in this particular experiment because the animals invariably died first.

Inorganic selenium compounds, when injected or administered orally, have been shown to be excreted quite rapidly by cats, rabbits (Smith et al., '37) and rats (Gortner and Lewis, '39); most of the selenium was eliminated within 2 weeks after the administration was discontinued. Smith et al. ('38) reported a greater retention of the naturally occurring organic selenium compounds than of Na_2SeO_3. In our experiments no such difference in retention of natural selenium compounds and Na_2SeO_3 was observed but our level of selenium supply was considerably lower (18 p.p.m.).

Munsell et al. ('36), in some preliminary studies on the retention of naturally occurring selenium compounds, indicated that there is a rather slow elimination of the selenium by the rat, but she did not establish how rapidly the major portions of this excretion took place, i.e., whether it was a constant amount or subject to considerable change. From a consumer's standpoint it should be interesting to know how fast and to what extent most of the elimination takes place. The following experiment was designed to supply some of this information.

EXPERIMENT

Previous data, obtained in this laboratory, indicated that the selenium concentration of the rats on a seleniferous diet showed a sufficiently close correlation within the groups to justify sacrificing a few animals from time to time as representatives of the group. In this experiment, then, we classified the animals into equivalent groups according to weight, sex and age, placed them on the seleniferous ration, and analyzed representative animals weekly as an index of the status of the whole group.

In the first series thirty adult rats received a ration having the following percentage composition: wheat no. 607 (30 p.p.m. of Se) 82; casein 10; salt mixture (McCollum) 1; pure

leaf lard 3; dried yeast [2] 2; and cod liver oil [3] 2. This ration contained approximately 25 p.p.m. of selenium and was fed ad libitum for a period of 4 weeks. The animals were then transferred to the stock ration having the following percentage composition: corn 32.5; wheat 37.5; rolled oats 20.0; alfalfa meal 5; and 1% each of cod liver oil, yeast, $Ca_3(PO_4)_2$, NaCl and $CaCO_3$ respectively.

Each week of the experiment, three representative animals were killed by decapitation and autopsied. Before being killed, the animals were brushed carefully to remove any adhering food particles. The livers were taken as one sample for selenium analysis, the rest of the carcass serving as another. After decapitation the liver was removed, washed in distilled water, drained on a pad of clean filter paper and dried on a watch glass. The alimentary tract was removed, split lengthwise, washed free of undigested food, and returned to the carcass. The whole carcass was then ground in a meat chopper and dried on a large watch glass. Analyses were made by the modified method of Robinson, Dudley, Williams and Byers as described in a previous report (Moxon, '37).

The second series was designed to be a repetition of the first but this time young growing rats were used instead of adult animals. Thirty rats weighing approximately 50 gm. each received the toxic ration for the 4-week period and were then transferred to the stock ration. A control group of ten rats was included in this series to serve as a basis for comparing the growth response.

The data presented graphically in figure 1 indicate that the animals continued to store selenium throughout most of the 4-week period, the amount stored reaching a maximum at the time that the toxic ration was removed. The young rats apparently stored greater amounts in their body tissues than

[2] Yeast foam powder manufactured by the Northwestern Yeast Co., Chicago, Ill.

[3] Cod liver oil containing 80 I.U. of vitamin D per gram, obtained from the National Oil Products Co., Harrison, N. J.

the adults, although the liver storage in the two groups proved to be roughly comparable. After the rats were transferred to the non-seleniferous stock ration there was a rapid fall in the concentration of selenium, especially in the livers. The quantity in this organ did not reach zero, however, and this may be taken as indicating that small amounts are bound too securely to be eliminated over a period of 6 weeks.

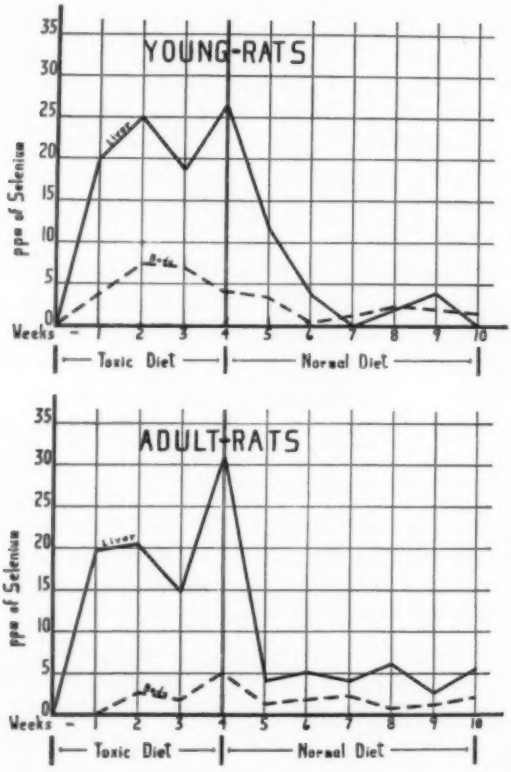


Fig. 1 Curves showing the concentration of selenium in the livers and the rest of the carcass of rats on the toxic diet, and the results obtained when animals are transferred to non-toxic rations.

Each point represents a composite sample from three rats, since the livers of individual rats offer too small a sample for accurate analysis.

The growth responses to the toxic and non-toxic ration are shown in figure 2. Subsistence on the toxic ration for 4 weeks had a considerably depressant effect upon the body-weight of both the young and adult rats.

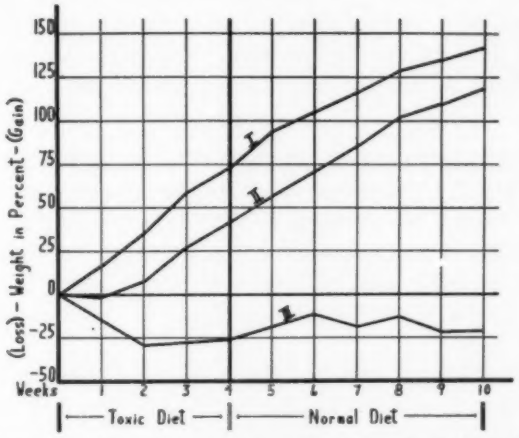


Fig. 2 Curves showing the percentage gain or loss in body weight of rats on the toxic rations as compared with the control group. (I) refers to the control group, (II) the young rats on the toxic diet and (III) the adult rats on the toxic diet.

DISCUSSION

The results obtained in this experiment indicate that at least for the rat, most of the selenium is eliminated within 2 weeks after the animals are transferred from a seleniferous wheat ration to a selenium-free diet. Some appears to be retained however, and this would account for the continued excretion of selenium which has been observed in cats (Smith et al., '38) to extend over a period of several months. If this condition exists in other species, then in spite of the fact that some of the naturally occurring selenium is retained for several months by the animal, from a quantitative standpoint much could be gained by placing the animals on a non-seleniferous ration for an appropriate period before slaughtering them. At least a large part of the selenium could be prevented from entering the human diet by this procedure.

The recent observation that bromobenzene (Moxon et al., '40) increases the selenium excretion from the animal body offers another possible procedure for making selenized animals safer for human consumption. Not enough is known as yet of the action of this compound in lowering the tissue concentration of selenium to warrant specification of details of such a method at the present time, but the observation opens a field worthy of more extensive investigation.

SUMMARY

1. The greater portion of the selenium absorbed by rats from naturally occurring seleniferous wheat was excreted when the animal was transferred to non-seleniferous stock ration. Some of the selenium was eliminated very slowly and appeared to be bound by the body tissues but the greater portion of the selenium appeared to be eliminated within 2 weeks.

2. Young rats appeared to store more selenium in the body tissues other than liver than old rats. Storage in the livers appeared to be similar.

3. Feeding of the toxic ration for only 4 weeks had a depressing effect upon the body weight of both young and adult rats.

LITERATURE CITED

GORTNER, R. A., JR., AND H. B. LEWIS 1939 The retention and excretion of selenium after the administration of sodium-selenite to white rats. J. Pharm. Exp. Ther., vol. 67, p. 358.

LEMLEY, RAY E. 1940 Selenium poisoning in the human. The Journal Lancet, Minneapolis, vol. 60, p. 528.

MOXON, A. L. 1937 Alkali disease or selenium poisoning. S. Dak. Agric. Expt. Station Bull. 311, p. 62.

———— 1941 Unpublished data.

MOXON, A. L., A. E. SCHAEFER, H. A. LARDY, K. P. DUBOIS AND O. E. OLSON 1940 Increasing the rate of excretion of selenium from selenized animals by the administration of bromobenzene. J. Biol. Chem., vol. 132, p. 785.

MUNSELL, H. E., G. M. DEVANEY AND M. H. KENNEDY 1936 Toxicity of food containing selenium as shown by its effect on the rat. U. S. Dept. Agric. Techn. Bull. 543, p. 21.

SMITH, M. I., B. B. WESTFALL AND E. F. STOHLMAN, JR. 1937 The elimination of selenium and its distribution in the tissues. U. S. Pub. Health Repts., vol. 52, p. 1171.

———— 1938 Studies on the fate of selenium in the organism. U. S. Pub. Health Repts, vol. 53, p. 1199.

IMPROVED DIETS FOR NUTRITIONAL AND PATHOLOGIC STUDIES OF CHOLINE DEFICIENCY IN YOUNG RATS

R. W. ENGEL AND W. D. SALMON

Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn

TWO PLATES (FIVE FIGURES)

(Received for publication February 17, 1941)

INTRODUCTION

In the investigations of vitamin B_6 in this laboratory symptoms of severe toxicosis frequently developed in rats fed purified diets supplemented with crystalline vitamins. The work of Griffith and Wade ('39 a) suggested that this toxicosis was a result of choline deficiency.

It is the purpose of this paper to report the effectiveness of various purified diets in producing choline-deficiency symptoms and to report the pathological changes accompanying these symptoms.

MATERIALS AND METHODS

Animals. Twenty-three-day-old male and female rats of the Steenbock and Wistar strains were kept in individual cages with raised screen floors. About 300 animals were used in these studies.

Diets. The diets listed in table 1 were used with varying degrees of success. The animals had continuous access to the diet except for a few hours each day when supplements were fed. Each animal received daily, in separate food jars, 20 μg.

each of thiamine chloride,[1] pyridoxine, and riboflavin, factor 2 concentrate equivalent to 1 gm. of liver and, in the case of rats on the fat-low diets, 0.1 ml. of corn oil. Vitamins A and D were supplied weekly in the form of beta-carotene and calciferol. Control animals each received 10 to 20 mg. of choline chloride per day.

TABLE 1

Percentage composition of the diets

DIET NO.	31 P	31 CP	31 PM	31 PMC	31 CPMC	31 F_2	6 L
Arachin [3]	18	17.7					
Peanut meal [2]			24	30	29.7		
Casein [3]				6	6	18	17.7
Sucrose	78	78	72	54	54	78	68
Salt [4]	4	4	4	4	4	4	4
Lard				6	6		10
Cystine		0.3			0.3		0.3

[1] Prepared from peanut meal according to Johns et al. ('16).

[2] Percolated with 61.5% alcohol by volume for 24 hours, followed by five 2-hour extractions with boiling 95% alcohol.

[3] Labco casein extracted four times (2 hours each) with boiling 95% alcohol.

[4] J. Biol. Chem., 1930, vol. 89, p. 199.

Technical procedures. The animals were killed by bleeding through the carotid vessels. Materials desired for routine microscopy were preserved in Bouin's fluid, embedded in paraffin and stained with hematoxylin and eosin. In some cases formalin-fixed sections of liver were sectioned in the frozen state and stained with scarlet red. Liver, kidney, and adrenal tissues were treated with Maximov's osmic acid stain for fat; the xanthydrol reaction (Romeis, '28) was used on

[1] The crystalline vitamins were generously supplied by Merck and Company, Rahway, N. J. The pantothenic acid fraction (factor 2 concentrate) used in this work was prepared as follows: 500 gm. of liver extract (Lilly no. 343) in 1500 ml. of H_2O was treated with 500 gm. of English fullers earth which was filtered out and washed with five 500 ml. portions of H_2O. The treatment was repeated five times with 250 gm. of fullers earth, the volume of the filtrate being kept up to 1 liter. The filtrate and washings were concentrated to 1250 ml., filtered and stored under Skelly Solve B at 3–5°C. until fed. In later experiments, it has been found that identical results are obtained by substituting calcium pantothenate (Merck) for the liver preparation.

some kidney and brain sections; and some kidney sections were treated with Mallory's ('38) aniline blue collagen stain.

EXPERIMENTAL RESULTS

Effectiveness of the diets

Diets 31 P, 31 CP, and 31 PM were equally effective in producing severe choline deficiency in all the animals. However, these diets were obviously inadequate in other essential food factors since control animals receiving 10 mg. daily of choline chloride made weekly gains of only about 5 gm. Diet 31 PMC produced severe symptoms in all the animals in 6 to 10 days. Control animals (20 mg. of choline chloride daily) remained normal and consistently gained about 3 gm. daily. Table 2 presents growth records of rats receiving this diet. Cystine added to this diet failed to stimulate growth.

TABLE 2

Weight and mortality record of rats fed diets with and without choline

DIET NO.	AVERAGE INITIAL WEIGHT	AVERAGE BODY WEIGHT GAIN IN GRAMS IN					
		6 days	7 days	8 days	9 days	10 days	14 days
	gm.						
	(20)	(20)	(20)	(18)	(16)	(11)	(1)
31 PMC	56	10	12	11	9	4	2
31 PMC+	(20)	(20)	(20)	(20)	(20)	(20)	(20)
20 mg. choline daily	54	18	22	25	29	34	48
31 CPMC +	(8)	(8)	(8)	(8)	(8)	(8)	(8)
20 mg. choline daily	49	15	20	23	28	33	49

The figures in parentheses indicate the number of rats surviving.

Diet 6 L produced gains of 2 to 3 gm. daily, but resulted in severe, acute symptoms in only about two-thirds of the animals. Diet 31 F_2 was used routinely in investigations concerning vitamin B-complex and fat deficiencies. On this diet about 50% of the male animals developed the typical symptoms and about half of these died. Females were somewhat less susceptible than the males on this diet. Considerable variations were also noted between different litters. Such litter or sex dif-

ferences were not noticed when the diets containing peanut meal were used.

It should be emphasized that the animals which made the best gains during the first 5 or 6 days on experiment usually developed severe symptoms earliest and failed most rapidly.

Description of the symptoms

Choline deficiency symptoms usually appeared within 6 to 10 days after the animals were placed on the experimental diets. Concomitant with drowsiness and inactivity, palpably enlarged kidneys and abdominal distention occurred. In severe, acute cases death resulted within 48 hours after the appearance of the first symptoms. Death was preceded by several minutes of extremely labored breathing, tremors, and coma. Extreme pain was evidenced by the fact that occasionally animals succumbed with their jaws set firmly in the cage wire. Circulatory impairment was indicated in the final stages by the loss of normal skin color, and lowered temperatures, particularly noticeable in the extremities.

Less common symptoms were diarrhea and eye hemorrhage. The eye hemorrhage appeared to originate in the ciliary vessels and spread into the posterior chamber. In a few instances the animals exhibited vertigo.

Blood samples from four rats on diet 31 PMC were pooled and the non-protein nitrogen was determined and found to be 247 mg./100 ml. of blood. Pooled samples of blood from two controls (20 mg. of choline chloride) had a non-protein nitrogen of 42 mg./100 ml.

Necropsy observations

Constant findings were enlarged, firm, bright red, hemorrhagic kidneys with thickened capsule, extremely pale, fatty livers (table 3), and atrophied thymus. The kidney weights of the most severe cases averaged over 100% more than the kidney weights of control animals fed the same diet plus choline. Hydrothorax was present in most cases, and less

TABLE 3

Kidney weight and liver fat in rats fed diets with and without choline

DIET NO.	NUMBER OF RATS	FRESH KIDNEY WEIGHT IN PER CENT OF MAXIMUM BODY WEIGHT	LIVER FAT [1]
		%	%
31 P	7	3.11	46.4
31 P + 10 mg. choline daily	8	1.33	15.6
31 CP	3	2.89	47.4
31 CP + 10 mg. choline daily	4	1.31	32.3
31 PM	4	2.68	45.6
31 PM + 10 mg. choline daily	4	1.32	24.0
31 PMC	4	2.61	49.5
31 PMC + 10 mg. choline daily	3	1.40	25.9
6 L	8	2.37	55.1
6 L + 20 mg. choline daily	8	1.14	11.4
31 F_2	8	2.37	...

[1] Per cent ether extract in the dry liver.

frequently, ascites was found. Foci of hemorrhage in the heart muscle, adrenal cortex, and lungs were frequently present. In many animals the lumbar and sacral lymph nodes were filled with blood. The spleen varied in appearance: in some animals it was extremely swollen and bright red; in others it was shrunken and quite pale. Severe congestion in the mesenteric vessels and in the descending aorta was also frequently seen.

Rats which survived an acute attack resumed growth and survived for from several weeks to several months. In such cases necropsy revealed a kidney surface severely pitted and scarred with connective tissue, and the capsule adhering to the surface. Animals in which the acute attack was less severe made an apparently complete recovery and showed only mild connective tissue scarring of the kidney surface.

Microscopic observations

Kidney. The renal hemorrhage originated in the arteriole beds in the periphery of the cortex. The efferent cortical vessels were primarily involved, but the glomeruli were also affected in the severely hemorrhagic condition. In the acute

fatal stages the kidney cortex became hemorrhagic throughout with disintegration and necrosis of the tubular system. In such kidneys the collecting tubules throughout the medulla were filled with blood, cellular debris, and hyaline masses. Extensive hemorrhage occurred between the capsule and the kidney surface. The capsule was thickened by connective tissue proliferation.

In animals which succumbed to the chronic form of the disease the tubular system of the kidney cortex was completely invaded by connective tissue with some calcification. The blood vessel walls were thickened in certain of the most severely scarred areas. The tubular system of the medulla retained its normal structure. Mild chronic cases presented only wedge-shaped areas of connective tissue invasion from the cortical surface inward, with tubules still normal in the adjoining areas.

Liver. The primary change in the liver was the uniform distribution of massive stores of fat in the liver cells. Occasionally the central veins were extremely distended and filled with blood.

Thymus. Parenchymatous atrophy and subsequent proliferation of fatty tissue resembled normal involution. Frontal sections through the organ showed only a small area of normal glandular tissue remaining.

Spleen. Cellular changes in the red pulp were variable. In some cases severe congestion was present and the red pulp was overcrowded with erythrocytes. In other cases the red pulp was atrophied and the whole organ shrunken. The connective tissue framework was usually normal.

Lymph nodes. The cortical substance sometimes was congested with erythrocytes and occasionally hemorrhage was present. Frequently, however, there were no abnormal changes.

Adrenals. Hemorrhage occurred in the arterioles of the capsule and between the cell columns of the cortex. It was most severe in the zona glomerulosa but occasionally it extended inward as far as the medulla. The cortical cell columns were often necrotic where severe hemorrhage had occurred.

Lungs. Usually severe congestion with varying degrees of edema and hemorrhage was present. Blood vessels were filled with blood and were severely distended.

Heart. Foci of hemorrhage and infiltration of white blood cells appeared in the myocardium in both right and left ventricles. Frequently there was extensive necrosis of the muscle fibers in the severely affected areas.

Xanthydrol reaction. Extensive deposits of needle-like crystals of xanthydrol in sections of brain and kidney indicated uremia.

DISCUSSION

The observations herein reported demonstrate conclusively that severe pathological changes result in young, growing rats fed diets deficient in choline. The pathological observations essentially confirm those reported by Christensen ('40).

Increase in kidney weight was found to be a reliable criterion for determining the effectiveness of various diets for producing choline deficiency. Approximately a 100% increase in kidney weight consistently resulted in either male or female rats fed the diets in which peanut meal was the sole or principal source of protein. This increase was about 65% in the animals fed diets containing casein as the principal source of protein.

Earlier work on kidney hemorrhage in rats fed purified diets has been adequately reviewed by Griffith and Wade ('40). Although it was concluded that cystine was the damaging agent, it is interesting that some of these workers were using diets which were low in choline. Griffith and Wade ('39 b, '40) and Griffith ('40 a) have shown that cystine added to the diet exaggerated choline deficiency symptoms and that either methionine or choline was protective. On the basis of these findings it would appear that the peanut meal diets used in the present investigations contained the sulphur-amino acids in ideal proportions for the production of choline deficiency. The sulphur-amino acid composition of these diets is not known, but the excellent growth obtained by the simple addition of choline chloride to diet 31 PMC indicated that these

amino acids were present in adequate amounts. Furthermore, the addition of cystine to these diets failed to stimulate growth.

Griffith ('40 b) has reported that male rats are more susceptible to choline deficiency than females. Sex and litter differences in susceptibility to choline deficiency were encountered in these experiments with diets containing casein as the sole protein, or in similar diets with 0.3% cystine added. Such litter and sex variations were not encountered, however, when the diets containing peanut meal were used. In experiments conducted to study mortality from choline deficiency on diet 31 PMC, seventy-nine out of eighty rats (forty females and thirty-nine males) died by the twelfth day of the experiment.

Since preliminary findings indicate that choline deficiency symptoms are in part manifested by high non-protein nitrogen in the blood, it is possible that the diets employed here would be ideal in studies concerning the production of experimental uremia.

The data presented here are of importance in relation to nutritional investigations where purified diets are employed. Subacute cases of choline deficiency could easily be overlooked since such animals appear quite normal and palpably enlarged kidneys is the only reliable symptom. Routine choline supplements to purified diets for the rat would appear essential on the basis of the present findings.

SUMMARY AND CONCLUSIONS

Diets were developed which consistently produced a fatal toxicity in young growing rats of either sex in from 6 to 10 days. The symptoms consisted of inactivity, abdominal distention, palpably enlarged kidneys, labored breathing, coma, and, in some cases, eye hemorrhage and vertigo.

Microscopic examination revealed severe hemorrhage in the kidney cortex, varying degrees of hemorrhage in the adrenals, lungs, myocardium and lymph nodes, massive deposits of fat in the liver cells and atrophy of the thymus. A high non-

protein nitrogen in the blood and a positive xanthydrol reaction indicated uremia.

Choline chloride (10 or 20 mg. per rat per day) prevented these symptoms. The advisability of routine choline supplements to purified diets in nutrition investigations with the rat has been emphasized.

The authors wish to thank Cornelia Stevens Flanagan for microtechnical assistance in this work.

LITERATURE CITED

CHRISTENSEN, K. A. 1940 Microscopic study of the effect of choline deficiency in young rats. J. Biol. Chem., vol. 133, p. xx.

GRIFFITH, W. H., AND N. J. WADE 1939 a Some effects of choline low diets. Proc. Soc. Exp. Biol. and Med., vol. 41, p. 188.

———— 1939 b Relation of methionine, cystine, and choline to renal lesions occurring on low choline diets. Ibid., p. 333.

———— 1940 Choline metabolism. II. The interrelationship of choline, cystine, and methionine in the occurrence and prevention of hemorrhagic degeneration in young rats. J. Biol. Chem., vol. 132, p. 627.

GRIFFITH, W. H. 1940 a Choline metabolism. III. The effect of cystine, fat, and cholesterol on hemorrhagic degeneration in young rats. J. Biol. Chem., vol. 132, p. 639.

———— 1940 b Choline metabolism. IV. The relation of the age, weight, and sex of young rats to the occurrence of hemorrhagic degeneration on a low choline diet. J. Nutrition, vol. 19, p. 437.

JOHNS, C. O., AND D. B. JONES 1916 The proteins of the peanut, arachis Hypogaea. I. The globulins arachin and conarachin. J. Biol. Chem., vol. 28, p. 77.

MALLORY, F. B. 1938 Pathological Technique. W. B. Saunders Company, Philadelphia.

ROMEIS, B. 1928 Taschenbuch der Mikroscopischen Technik. R. Oldenbourg, Munchen and Berlin.

PLATE 1

EXPLANATION OF FIGURES

1 Kidneys from litter mate rats after 7 days on diet 31 PMC (upper) and diet 31 PMC + 10 mg. choline chloride daily (lower). The dark surface of the upper kidney is due to hemorrhage in the cortex. Also notice the size difference. × 2.5.

2 Choline deficiency. Section of kidney cortex. Notice the accumulation of blood in the spaces surrounding the necrotic convoluted tubules, and in the glomeruli. × 150.

3 Higher magnification of the outlined area in figure 2. Notice the extreme congestion in Bowman's capsule and the rupture of its wall, also the necrotic tubules surrounded by the red blood cells. × 320.

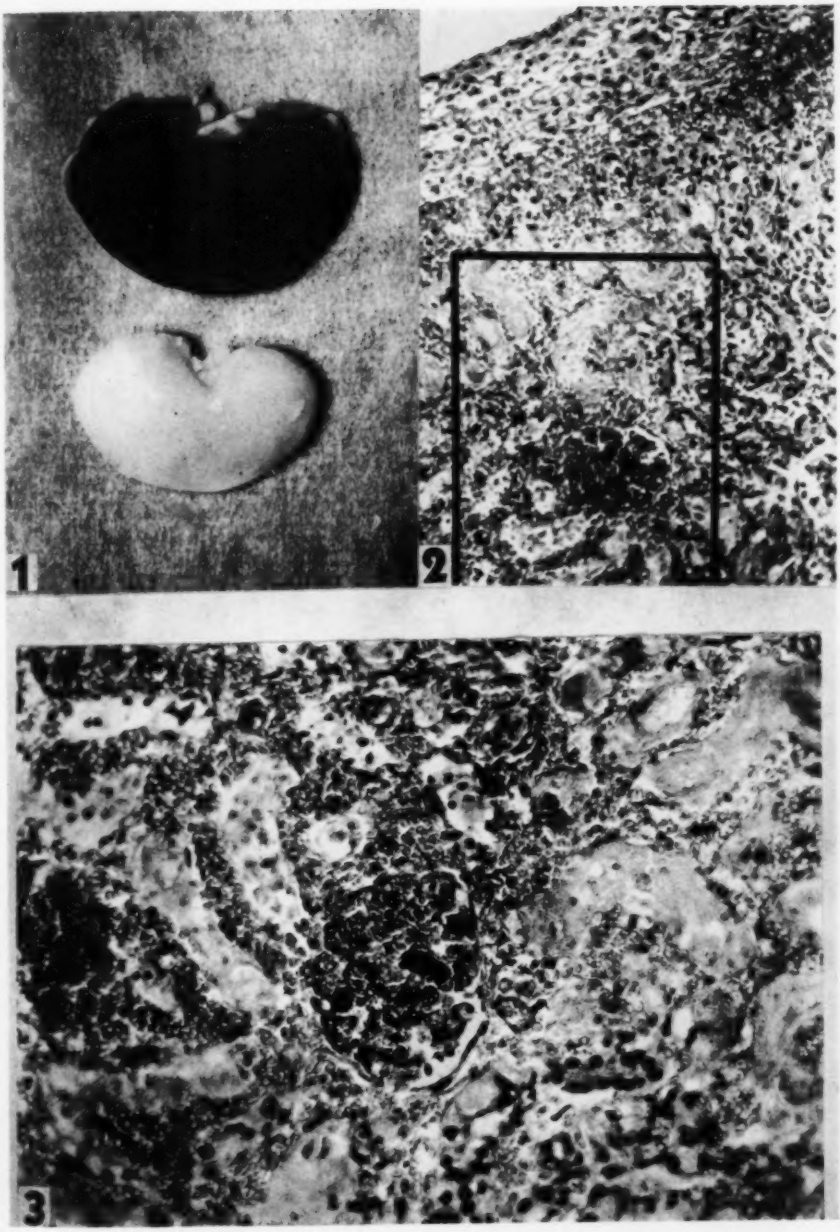
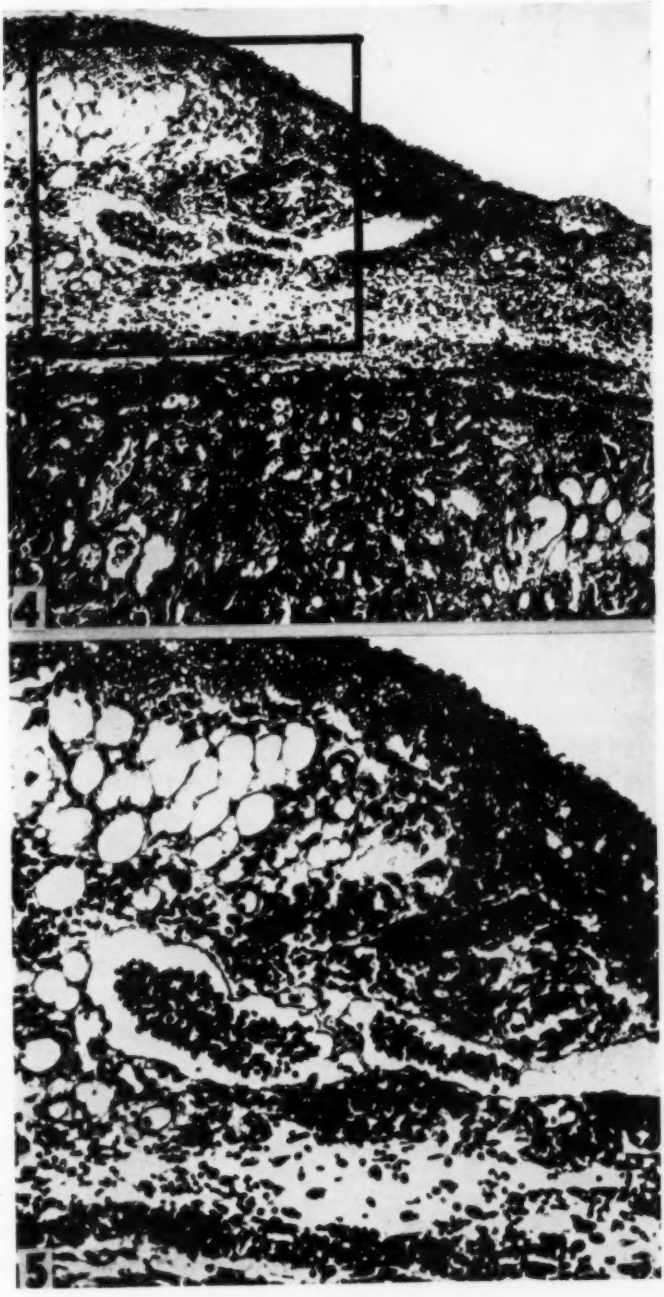

1
2
3

PLATE 2

EXPLANATION OF FIGURES

4 Choline deficiency. Section of kidney capsule (upper half) and kidney cortex (lower half). Notice the extremely thickened capsule. The convoluted tubules in the cortex are completely destroyed. × 115.

5 Higher magnification of the outlined area in figure 4. Notice the unusually large blood vessels in the capsular connective tissue and the accumulation of red blood cells in the extravascular tissue. × 265.


4
5

THE GROWTH CURVE OF THE ALBINO RAT IN RELATION TO DIET

THEODORE F. ZUCKER, LILIAN HALL, MARGARET YOUNG AND LOIS ZUCKER

Department of Pathology, College of Physicians and Surgeons, Columbia University, New York

FOUR FIGURES

(Received for publication January 28, 1941)

Studies in nutrition as carried out on laboratory animals, especially rats, have dealt largely with deficiency states. Deficiencies manifest themselves either in more or less specific symptoms or in a growth deficit below the normal growth for the given age. Deficits in growth can be measured against controls but are more frequently established by growth resumption when the deficiency is remedied. As our knowledge progresses and the requirements of experimental work become more rigorous the question often arises as a practical issue: "What is normal or optimal growth or what should be the course of growth in the rat strain employed, when there are no deficiencies?" Control groups of sizes commonly employed usually give the answer but do not always completely satisfy all the requirements. The collection of data and their analysis here presented were not undertaken with a view to the general biology of growth but arose out of the practical necessity of knowing more of the growth performance of the rat.

EXPERIMENTAL

In table 1 will be found the mean weights from 4 weeks (age of weaning) to 17 weeks of all the males and females introduced into our breeding colony in 1936 and 1937, together

with the standard deviations and coefficients of variation. In figure 1, curve 2 represents the 1936 females, with some additional data up to 50 weeks of age and on pre-weaning growth. The individual points in the 17–50 week period are based on small numbers, and ages as well as weights are averaged, since during this period the females were bred repeatedly and were weighed only occasionally between pregnancies. We have only a few pre-weaning data on these 1936 and 1937 rats. More extensive recent data show that pre-weaning growth in our colony is properly represented by these few earlier data. The number of young each mother was allowed to raise ranged

TABLE 1

Body weight and variability of breeding animals on stock diet, 1936 and 1937 combined.

WEEKS	FEMALES N = 311			MALES N = 42		
	M	σ	CV	M	σ	CV
4	51.4	4.5	8.8	61.0	5.2	8.5
5	72.8	6.7	9.2	87.6	8.1	9.3
6	92.4	9.6	10.4	115.4	13.2	11.4
7	107.0	11.3	10.5	138.6	18.3	13.2
8	119.0	13.1	11.0	164.1	22.9	14.0
9	130.0	14.4	11.1	185.9	24.0	12.9
10	140.0	14.9	10.6	204.8	24.5	12.0
11	148.7	14.9	10.0	221.3	23.9	10.8
12	156.9	14.8	9.6	236.6	23.2	9.8
13	163.0	14.8	9.1	249.6	23.8	9.5
14	168.9	15.5	9.2	261.7	24.4	9.3
15	173.2	15.1	8.7	271.4	25.9	9.5
16	178.5	17.2	9.5	280.6	25.8	9.2
17	182.8	18.3	10.0	288.1	27.9	9.7

$$\sigma = \sqrt{\frac{\Sigma d^2}{N-1}} \qquad CV = \frac{\sigma}{M} \times 100.$$

The mean values (M) were obtained for each age on the same 311 females and the same 42 males. The age tabulated is the actual age to within a day of each individual rat. Weights were determined to the nearest gram.

The standard deviations (σ) and coefficients of variation (CV) are intended to characterize these particular data only. They do not characterize the colony as a whole because the breeding stock animals are selected at weaning so that the weights cluster around chosen means. The coefficients of variation of the weaning weights of the total (unselected) population during 1936 are: male CV = 15.6, N = 603; female CV = 14.5, N = 676. We do not know whether the peak in variability at about 8 weeks, with males more variable than females, found for the breeding stock would also be found for the total colony output (see Sherman and Campbell, '34).

from four to eight, seven being by far the most frequent, 75% of the total being either six or seven. The animals were fed upon our regular stock diet, with no special supplements at any time. This diet contains 15% each of ground yellow corn, ground hulled barley, ground shelled oats, ground whole wheat and soy bean meal. It contains 10% each of meat scrap and whole milk powder, 2% each of alfalfa leaf meal and sodium chloride, and 0.5% each of calcium carbonate and molasses (as a binder in pellet making). Essentially this form of diet has been in use in this laboratory since 1932. In 1936 it became available in pellet form, and has been found convenient and satisfactory in our own colony as well as in several others in this institution. The subject matter of table 2 discussed below indicates that the stock diet as far as growth is concerned has no properties different from the diets of the other investigators there mentioned.

DISCUSSION

Formulation of data. Figure 1 is a comparison of our growth curve with several others in the literature. Curve 1 represents the growth of female rats of The Wistar Institute colony; instead of using the smoothed curve of Donaldson's monograph ('24) we have taken the original data which appear only in the paper of Donaldson, Dunn and Watson ('06). For a time this was considered standard rat growth and was used by Robertson, Brody, and Pearl as the basis for equations in the theoretical study of growth. Its salient characteristic is a sigmoid form between the ages of weaning and adult life, and it was this feature, with its point of inflection located in the post-weaning period as shown in the graph, around which the theoretical considerations largely centered. Referring to these generalizations, in presenting the results of improved diets Osborne and Mendel ('26) say: "The 'curves of growth' . . . may not be in harmony with some of the current interpretations of growth." Their 1926 data were nearly free of the post-weaning point of inflection, and what is left of it occurs too early to fit in with the interpretations mentioned. Curve 3

of figure 1 represents the most recent growth curve of the Osborne-Mendel colony (Mendel and Hubbell, '35).

Curves 2 and 3 are representative of the greatly simplified shape of post-weaning rat growth obtained on the newer stock diets. All growth curves have a point of inflection, but this is now completely transferred to the pre-weaning period as illustrated in figure 1. The simple form, with constantly decreasing slope, with no trace remaining of the strongly marked sigmoid

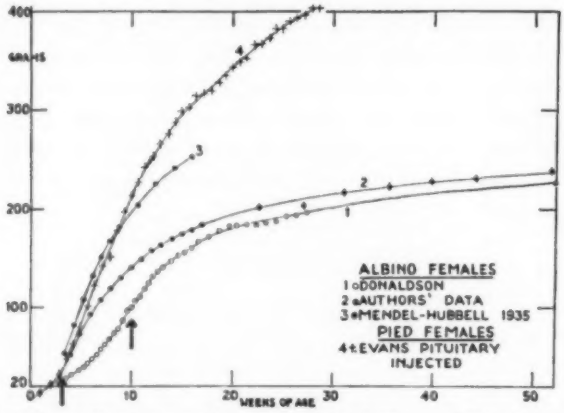


Fig. 1 Rat growth curves—ordinary (linear) plotting. Authors' data represent 167 female rats reared for breeding purposes in 1936. Arrows indicate the point of inflection in the Donaldson data, at 10 weeks, and in the authors' data, at 3 weeks. The point of inflection is the point where the curve is steepest.

form of the older data, seemed to justify the attempt to transform it into a straight line function of variables based on time and weight. This was done simply for greater ease of interpretation of data and comparison of results. Such an empirical transformation, if it leads to a simple equation, is always useful but need not have, as such, any reference to theoretical interpretation or to the mechanism of the growth process.

It was found that, if the logarithm of the weight is plotted against the reciprocal of time, a good straight line results. The formula for this straight line is:

$$\log W = \frac{-k}{t} + \log A,$$

W being the weight at time t. The equation is a very simple one, containing merely the minimum number of constants necessary to define a straight line; A is the weight approached asymptotically [1] in the adult animal (log A being the intercept of the straight line), and k is the slope of the line which characterizes the rate of growth ($k = \frac{\log W_2 - \log W_1}{1/t_1 - 1/t_2}$). On semi-log paper with a time axis of reciprocals (4 weeks = 0.25, 5 weeks = 0.20,

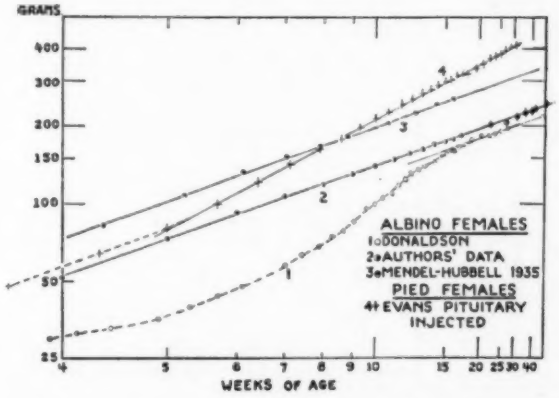


Fig. 2 Log-reciprocal plotting (logarithm of W against reciprocal of time) of the data of figure 1. For clarity the proportions of this graph resemble those of figure 1, and background lines are omitted.

etc.) the data can be plotted directly as obtained, with no preliminary calculations or adjustment of constants as is the case in the application of most other proposed equations, and of all other proposed equations covering this time range. Figure 2 is such a plot of the data of figure 1. Figure 3 shows our 1937 female data, and the averaged 1936 and 1937 data on males,

[1] The asymptotic growth formula may at first sight appear disturbing. It is however by no means irrational particularly in the rat where closure of the epiphyses of the long bones happens very late in life (Dawson, '25), if it ever happens so completely that further growth in length is excluded. Due consideration should be given to the fact (McCay et al., '39) that after 1000 days of retarded growth, rats are still able to resume growth. Because of the nearly universal respiratory infections after middle life, it is difficult to obtain valid data to prove or disprove asymptotic growth.

plotted on the log-reciprocal paper used routinely in our laboratory. Both with our data and with those of Mendel and Hubbell the agreement with the proposed formulation is excellent. This is true although Mendel's animals are quite a bit

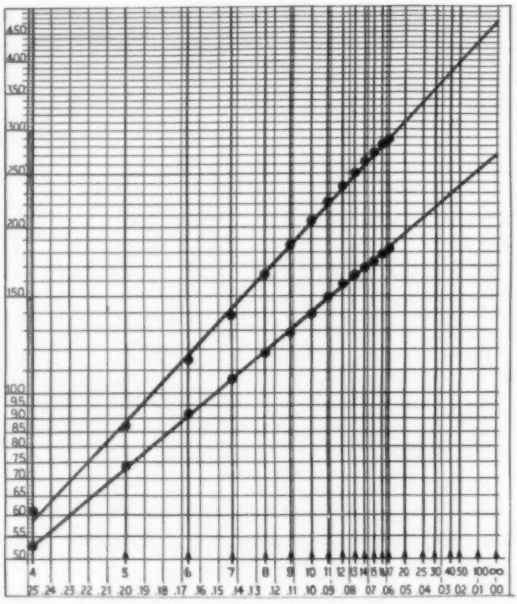


Fig. 3 The lower curve gives the mean values for 144 females reared for breeding purposes in 1937. The upper curve represents the weights of forty-two males reared during 1936 and 1937. The graph paper is that used for routine records. The lines are the best fit to the combined 1936 and 1937 data, obtained by the method of least squares, which calculation also gives directly the values for k and A. Fitting can also be done by eye; A can then be read off the fitted line at infinity on the time scale, as indicated in the figure, and k can be calculated from any two points on the line, using the formula for k given in the text.

larger, having been selectively bred for size, as a part of the program of developing large rats, referred to in a number of papers from Mendel's laboratory. The size difference of genetic origin (Mendel's rats and ours are of the same original strain) has no effect on the shape of the growth curve, both

following the log-reciprocal formula equally well. The only difference is in the constant A.

In figure 2 we have illustrated the occurrence of rats of two different inherent average sizes with the same growth k. It is now of interest to ascertain to what extent k and A will vary in other rat colonies. We have found in the literature data on growth of rats which will throw some light on this point. Some of these have been collected in table 2. It will be seen that for albino rats both the male and female k's vary little from their

TABLE 2

Constants of log-reciprocal equation.

	FEMALES			MALES			k_{male}
	Age range	k	A	Age range	k	A	k_{female}
Authors' data [1, 2]	4–50	2.84	270	4–17	3.65	470	1.29
Mendel-Hubbell ('35),[2] 1935 rats	3–16	2.84	380	3–39	3.73	650	1.31
Smith-Bing ('28)	3–33	2.76	316	3–33	3.67	548	1.33
Macy et al. ('27) — group labelled "stock diet" [3]	4–30	2.86	278	4–30	3.66	484	1.28
Freudenberger ('32)[3]— albinos	3–64	2.80	282	3–64	3.70	440	1.32
King ('15)[1, 3, 4]	4–70	2.98 2.84	251	4–70	3.73	360	1.25 1.31
Sperry-Stoyanoff, Series III ('34)[5]				3–22	3.68	476	

[1] Females known to have been bred regularly and weighed between pregnancies.

[2] The original observations were not available; the points used in fitting were read off the published smoothed curves.

[3] Rat groups a year or more old present a problem with regard to sickness and death, as mentioned below in connection with the Evans data. Freudenberger states that a not negligible number of abnormal weights were included in his mean values. The end of the curve therefore falls off the log-reciprocal line just perceptibly in the males, and quite obviously in the females. King applied a correction in her published figures. We have likewise done so.

[4] The King data are among the earliest. The diet—"selected table scraps"—could, however, very well be good by current standards, and we have included the data because of the generally excellent fit to the equation. The points have a very unfortunate distribution on a log-reciprocal plot because of the large (30 day) interval between weighings. The decision between 2.98 and 2.84 for the female k rests on one point (the 30 day weight). The A value is not affected. We are inclined to accept the figure 2.84, particularly since the male data show no peculiarity.

[5] Detailed growth data kindly supplied by Dr. Sperry.

means. The ratio $\frac{k_{male}}{k_{female}}$ is constant within about 3% of the mean.

The thought that the formula represents the inherent qualities of the growth curve is further substantiated in the case of animals artificially stimulated beyond their normal growth. Curve 4 in figure 1 and in figure 2 relates to data of Evans ('23–'24) on female rats of the pied Long-Evans strain, injected daily with anterior pituitary substance. In Evans' table of individual weights a certain number of deaths are recorded and a few other cases of definite loss in weight. Since we are discussing normal growth, we have omitted from the mean values all weight data indicative of either definitely abnormal conditions or approaching death. This procedure brought about an improvement in the smoothness of the curve. These animals, similar in weaning weight to the rats of curve 2, outgrow, under the influence of the pituitary substance, not only the animals of curve 2 but also the much larger ones of curve 3. In the log-reciprocal plot, after a short latent period, they also follow a straight line. The uninjected controls follow a course essentially a straight line prolongation of the latent period (fig. 2).

It is apparent therefore that neither size nor growth rate appears to affect that growth property of rats which causes them to increase their weight according to the scheme expressed by our empirical formula, as long as they are on diets such as the present-day good stock diets free from known growth inhibiting deficiencies.

Individual growth curves. This remarkable adherence to a simple formulation by so many different colonies, with apparently all differences in growth among albinos of the same sex controlled by a single variable (A), raises a question as to the growth curves of individual rats. The data given so far are all based upon means of large numbers. What is the course of the individual curves? Is the constancy of k due to the averaging of large numbers of different k's with essentially the same distribution in each colony, or is this a feature of the individual curves also?

It has been found that many individual curves show excellent adherence to a log-reciprocal line of slope 2.84. The error on points which make up an individual rat's growth curve is small enough so that it can be fairly said that most of the animals adhere satisfactorily to the formula with this particular slope. Since size (as represented by A) varies over a certain range, this results in a series of parallel lines on log-reciprocal paper. When k, the slope, and log A, the intercept, are calculated by least squares for individual rats, a certain range of k, as well as of A, is observed. These deviations from the mean slope present a real problem, for some deviations in the calculated slopes would be expected (experimental error) even if the true slopes were all identical. The limitations of the least squares method and the difficulty of error calculations on such data become very apparent. We are not able to say that in the case of individual albino rats we have been able to establish the existence of several different k values; the balance of evidence goes to show that the apparent differences are due to error in the original data, and that albino rats of our colony adhere to a single slope with moderate error. An actual range of size (i.e., A value) beyond the experimental error seems to be established. This view on albino rat growth is strengthened by the fact that a strain bred from hybrids (Long-Evans strain) shows in our hands an easy division into individuals of small initial size with large slope, and large initial size with small slope. This is at least in part verified by the study of various data on Long-Evans rats from several other laboratories; the k's for various colonies are all different, and even our own Long-Evans colony k is not quite the same from year to year.[2] It is plausible enough from a

[2] It is interesting to note that the identity of the ratio of male to female k from colony to colony holds for Long-Evans rats as well as albinos. This constancy is hardly accidental, and can probably be taken as corroboration of the thought that the formula gives a useful picture of the course of growth in the rat. It also means that in individual Long-Evans colonies the male k changes pari-passu with the female k. There are enough data on the distribution of coat color patterns in the various experimental records on Long-Evans rats so that on mere inspection it seems evident that one whole colony (males and females) may differ characteristically in its genetic composition from another.

genetic point of view that the hybridization of two very different strains (as in Long-Evans rats) should leave the haphazardly bred colony with at least some representatives of two initially different growth slopes. It is admitted that this may possibly also occur in the albino rats, but if so the differences must be small, since it has not been possible so far to establish proof for such a condition.

During the time these data have been collected we have found a few individual rats which obviously do not follow our growth curve. In some of these food consumption had been recorded and it was found that subnormal growth was accompanied by subnormal food intake. Sooner or later unmistakable signs of pathological state (usually respiratory infection) became evident. On several occasions an unusually high infant mortality has developed in the colony, passing off again without a definite cause being ascertained. At such times the surviving rats have shown individual deviations from the growth curve larger than usual during the early part of the curve with good adherence later on. Just recently we have for the first time (in over 3 years) had an isolated group of ten rats show an unprecedentedly high growth slope from 4 to 8 weeks. This might be due to undernutrition before or during the weaning time with a modified realimentation. We have, however, no concrete explanation to offer. All these exceptions taken together have not enough weight to lessen materially our belief that *normal* rat growth follows the scheme here outlined, but have rather stimulated our interest in detecting and eliminating abnormalities.

Spontaneous realimentation. We have cited a number of instances where informed efforts to eliminate deficiencies have led to post-weaning growth curves which satisfy the conditions of the proposed formula even though the intrinsic size of the animal varies. Deviation from the formula with experimentally varied deficiencies will be dealt with in future reports. However, there are in the literature numerous examples (Donaldson and others) where growth according to the formula has not been achieved and it seems likely that the reason

for this deviation is to be found in the existence of dietary deficiencies. The Donaldson curve has already been presented in figure 1 and figure 2. These data were obtained at a time when little was known concerning nutritional requirements. They illustrate a common type (though not the only one) of deviation from formula growth. On linear paper the curve is sigmoid, i.e., a slow initial rate of growth is followed by a larger rate after which again the rate decreases. The same features are illustrated in many of the early Mendel rats and the Greenman and Duhring data (Donaldson, '24). The diets employed allowed the animals to grow and propagate, and so were satisfactory as stock diets. They were, however, distinctly inferior to the type of diet most investigators now choose, in line with the thought expressed by Freudenberger ('33) that ". . . it is well known that the rat thrives much better on our more modern diets." The particular peculiarity of these curves is that the rate of growth increases temporarily after weaning, while on the newer (better) diets the rate falls off continuously from 4 weeks on. Judging by growth rate the Donaldson animals are in a better nutritional condition after about the tenth week than from the third to the seventh week. This improvement with no change in diet resembles the finding of high growth rates shown by Osborne and Mendel ('16), Clark and Smith ('38), Jackson ('36, '37) and others to be characteristic of the realimentation or recovery curve obtained when rats are changed from a poorer to a better diet. Similarly in the log-reciprocal plotting (fig. 2) the final k value of Donaldson is "normal" but straight lines fitted to intermediate portions of the curve would indicate unusually high k values (recovery), while the initial portion of the curve has a very low slope. The "normal" final k value together with the intermediate high linear rate or high k values as against the low initial rate or k indicates that actually the diet at later ages satisfies the growth requirement better than at an early age. This is not inconsistent with the many findings to the effect that signs of deficiency are more easily produced at early ages or that requirements in the young are particularly

critical. We give this explanation for the sigmoid feature of deficient growth very tentatively. We give it at all only because it appears that the subject matter is of considerable interest. We have in mind particularly the many references to "preadolescent growth spurt" etc., as a normal or unavoidable phenomenon in growth.

With moderate or mild deficiencies a post-weaning curve may result which in linear plotting is only doubtfully sigmoid or not at all so and still does not fit the proposed formula. Diet

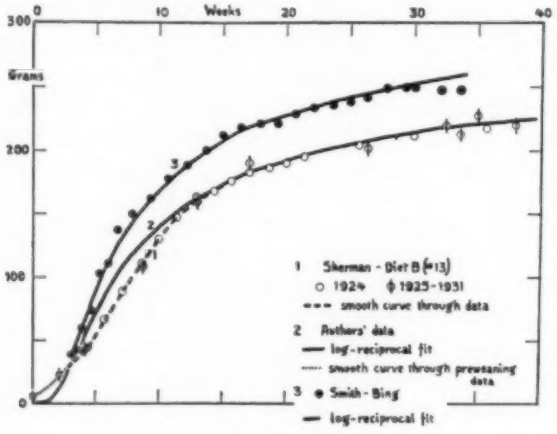


Fig. 4 See text.

B (no. 13) of Sherman is considered a normal diet and is used for studying the effect of supplements. Figure 4, curve 1, shows the growth on Sherman's diet B ('24, '25, '26, '31) compared with that on our stock diet (curve 2) and that of Smith and Bing ('28), already mentioned in table 2. These animals are all females of the same original strain; our colony is an offshoot of Sherman's, and his in turn an offshoot of the Osborne-Mendel colony, which is represented by the rats of Smith and Bing. Smith and Bing's rats were selectively bred for size; Sherman's and ours were not, as is demonstrated by the identical final size. The difference between diet B and that

of Smith and Bing is that the latter has a lettuce supplement, substitutes calcium carbonate for some of the sodium chloride, and gives nursing mothers a supplement of yeast. A comparison of the latter two curves shows clearly that growth according to our equation can be achieved by judiciously supplementing a deficient diet. Rats raised for generations upon Sherman's diet B and on our stock diet apparently show no significant differences in weight very early in life and as adults. The deficit during the period from 4 to 13 weeks is entirely in line with what has been said above concerning the apparent spontaneous realimentation on quite inadequate diets.

Pre-weaning growth. We have emphasized that the proposed formulation applies from the 4 weeks weaning time onward as far as suitable data are available. This is the phase of growth, auxano-differentiation of Huxley (Huxley, '32, p. 148) in which the weight of the animal increases essentially on the basis of completed development of its parts. The earlier phase (conception to weaning) involves largely the differentiation of the various organs and organ systems (Huxley's histo-differentiation). The approximate location of the transition from histo- to auxano-differentiation in the rat at 3 to 4 weeks rests on a variety of evidence (Freudenberger, '32: definite change in proportion of measurements ceases at about 3 weeks; Donaldson, '24: fully functional state of sense organs sets in at this time; Jackson, '32: extended discussion; Cole, '37: successful hormonal stimulation of sex organs in female is possible any time after 3 weeks but not before). We would therefore, both a priori and on the basis of Huxley's discussion, not expect a given simple expression to fit both phases of growth. The log reciprocal formula includes a point of inflection at about 3 weeks, and the curve turns in the right direction. In harmony with what has been said concerning histo- and auxano-differentiation it does not, however, give even an approximation to the data from birth to 3 weeks, as can be seen graphically in figure 4. All this has no bearing on the practical objectives as outlined in this report, but involves

a number of interesting problems along the lines laid down by Huxley.

In our experience the weight at 3 weeks often falls on the log reciprocal graph but at other times even the 4-week weight gives not as good a fit as later on. In many respects it is unfortunate that animals are usually selected for assignment to experimental groups at an age associated with the marked transition character described above as well as with the dietary changes consequent to weaning.

Significance of the proposed formulation. In this discussion we are not interested in the question raised by Osborne and Mendel ('26) years ago whether maximal growth is most advantageous to the animals, etc., nor are we attempting to define "normal" growth. The basic thought we are developing in this and several succeeding reports is that on diets adequate for best growth, i.e., diets in which no experimental restriction is placed on the natural optimal growth inherent in the species, we find that relations of weight to time, weight to food intake and to relative weights of parts of the body, etc., are simpler in form than on deficient diets where natural growth is inhibited. If effects of deficiencies are to be elucidated an unhampered norm should first be worked out. We merely point out that removal of all known deficiencies which arbitrarily hamper growth would be expected to lead to simpler conditions as far as growth is concerned, and does in fact produce a simpler scheme of weight increase.

While we are not attempting any rationalization of the growth formula here proposed, it may not be out of place to call attention to the fact that it is in line with two well-known concepts regarding growth which recur constantly in the literature on the general biology of growth. These are very succinctly formulated by Huxley ('32, p. 6). "One essential fact about growth is that it is a process of self-multiplication of living substance, i.e., that the rate of growth of an organism growing equally in all its parts is at any moment proportional to the size of the organism. The second fundamental fact about growth is that the rate of self-multiplication slows down with

increasing age (size)." The first of these concepts is expressed in our formulation by logarithmic plotting of the weight, the second by the reciprocal time axis.

SUMMARY

1. The growth of rats on present-day good stock diets is characterized by a progressive decrease in gain for each successive time interval after weaning.

2. Such data can be conveniently plotted as a straight line if the logarithm of the weight and the reciprocal of time are used as coordinates. The formula is:

$$\log W = \frac{-k}{t} + \log A.$$

These findings are corroborated by numerous data of other investigators.

3. Neither natural variation in size of the animal nor artificial stimulation of growth rate causes a deviation from the empirical formula of growth. While inherent size of rats varies considerably, the slope of the plot (k of the formula) varies but little for each sex in albino rats. In all cases where data for males and females are available, the ratio of k for males and k for females is constant within 3%.

4. Deviations on diets suboptimal for growth are discussed.

LITERATURE CITED

CLARK, M. F., AND A. H. SMITH 1938 Recovery following suppression of growth in the rat. J. Nutrition, vol. 15, p. 245.

COLE, H. H. 1937 Superfecundity in rats treated with mare gonadotropic hormone. Am. J. Physiol., vol. 119, p. 704.

DAWSON, A. B. 1925 The age order of epiphyseal union in the long bones of the albino rat. Anat. Rec., vol. 31, p. 1.

DONALDSON, H. H. 1924 The Rat. Memoirs of The Wistar Institute of Anatomy and Biology, No. 6.

DONALDSON, H. H., E. H. DUNN AND J. B. WATSON 1906 A comparison of the white rat with man in respect to the growth of the entire body. Boas Anniversary Volume, pp. 5–26. G. E. Stechert & Co., New York.

EVANS, H. M. 1923–1924 The function of the anterior hypophysis. Harvey Lectures.

FREUDENBERGER, C. B. 1932 A comparison of the Wistar albino and the Long-Evans hybrid strain of the Norway rat. Am. J. Anat., vol. 50, p. 293.

———— 1933 Variability in body length, body weight, and organ weights of the rat. Anat. Rec., vol. 56, p. 47.

HUXLEY, J. S. 1932 Problems of relative growth. Methuen and Co., London.

JACKSON, C. M. 1932 Structural changes when growth is suppressed by undernourishment in the albino rat. Am. J. Anat., vol. 51, p. 347.

———— 1936 Recovery in rats upon refeeding after prolonged suppression of growth by dietary deficiency of protein. Am. J. Anat., vol. 58, p. 179.

———— 1937 Recovery of rats upon refeeding after prolonged suppression of growth by underfeeding. Anat. Rec., vol. 68, p. 371.

KING, H. D. 1915 The growth and variability in the body weight of the albino rat. Anat. Rec., vol. 9, p. 751.

MACY, I. G., J. OUTHOUSE, M. L. LONG AND A. GRAHAM 1927 Human milk studies. I. Technique employed in vitamin studies. J. Biol. Chem., vol. 73, p. 153.

MCCAY, C. M., L. A. MAYNARD, G. SPERLING AND L. L. BARNES 1939 Retarded growth, life span, ultimate body size and age changes in the albino rat after feeding diets restricted in calories. J. Nutrition, vol. 18, p. 1.

MENDEL, L. B., AND R. B. HUBBELL 1935 The relation of the rate of growth to diet. III. A comparison of stock rations used in the breeding colony at the Conn. Agr. Exp. Station. J. Nutrition, vol. 10, p. 557.

OSBORNE, T. B., AND L. B. MENDEL 1916 Acceleration of growth after retardation. Am. J. Physiol., vol. 40, p. 16.

———— 1926 The relation of growth to diet. I. J. Biol. Chem., vol. 69, p. 661.

SHERMAN, H. C., AND H. L. CAMPBELL 1924 Growth and reproduction upon simplified food supply. IV. Improvement in nutrition resulting from an increased proportion of milk in the diet. J. Biol. Chem., vol. 60, p. 5.

———— 1934 Observations on growth from the viewpoint of statistical interpretation. Proc. Nat. Acad. Sci., vol. 20, p. 413.

SHERMAN, H. C., AND F. L. MACLEOD 1925 The calcium content of the body in relation to age, growth and food. J. Biol. Chem., vol. 64, p. 429.

SHERMAN, H. C., AND E. J. QUINN 1926 The phosphorus content of the body in relation to age, growth and food. J. Biol. Chem., vol. 67, p. 667.

SHERMAN, H. C., AND L. E. BOOHER 1931 The calcium content of the body in relation to that of the food. J. Biol. Chem., vol. 93, p. 93.

SMITH, A. H., AND F. C. BING 1928 Improved rate of growth of stock albino rats. J. Nutrition, vol. 1, p. 179.

SPERRY, W. M., AND V. A. STOYANOFF 1934 Effects of long-continued cholesterol feeding in rats. J. Nutrition, vol. 9, p. 131.

GROWTH AND CALCIFICATION ON A DIET DEFICIENT IN PHOSPHATE BUT OTHERWISE ADEQUATE

THEODORE F. ZUCKER, LILIAN HALL AND MARGARET YOUNG

Department of Pathology, College of Physicians and Surgeons, Columbia University, New York

FOUR FIGURES

(Received for publication January 28, 1941)

The finding that experimental rickets can be produced in rats came not by systematic design of diets for this purpose, but through the examination of bones of animals which had been kept on diets developed for other much more general purposes. Of the two pioneer diets no. 84 of Sherman and Pappenheimer ('21) was simple in its makeup but had marked multiple deficiencies resulting in very little growth and was uncertain in its action, while no. 3143 of McCollum et al. ('21) gave more reproducible results and fair growth but was quite complicated. A simplifying step was made when Steenbock and Black ('25) published their diet no. 2965 which was easy to compound, satisfactory as to reliability and also gave better growth than no. 84. For purpose of bringing about rickets for ordinary curative experiments it has stood the test of time and is justifiably more generally used than any of the other available diets. For certain purposes, however, qualities not shared by any of the diets mentioned are desirable. This is attested to by Jones ('38), by Schneider and Steenbock ('39), and by others.

A number of diets deficient only in phosphate have been devised, most of them containing less phosphorus and producing a more marked rickets than the traditional rachitogenic

diets. The present report deals with a diet deficient only in phosphate (no. 803), which, however, produces rickets to about the same extent as no. 2965. This allows a direct comparison of the two diets, the intensity of the rickets production and the calcium content being the same. The similarity in rickets producing quality is probably the resultant of a higher available phosphorus content in no. 803 accompanied by better growth. In the ration no. R14 of Schneider and Steenbock ('39), which with phosphate supplements produces excellent growth, the phosphorus is very low and calcium also proportionately lower. The interesting and rather unexpected results recorded by these authors show that comparison with no. 2965 is quite a bit more complicated.

EXPERIMENTAL

Rats from our albino colony were weaned at 4 weeks of age and placed upon various rations: no. 2965, our diet no. 803 and our stock diet (see preceding paper, Zucker et al., '41, for composition); no. 2965 and no. 803 supplemented with 2.0% KH_2PO_4 (an amount shown to give optimal results); all three diets supplemented with adequate vitamin D; no. 803 supplemented with both phosphate and D.

The composition of diet no. 803 is, in per cent: starch, 65; cottonseed flour, 20; casein, 6; egg albumen, 3; NaCl, 1; cottonseed oil containing carotene, 2; $CaCO_3$, 3. The diet contains 0.28% of phosphorus, of which 52% is in the form of phytin. The phosphorus content is about the same as average values for no. 2965, but the phytin fraction is lower than is found in analyses of most samples of no. 2965 according to Harris and Bunker ('35). The carotene being mixed into the diet was put at a probably unnecessarily high level equivalent to 750 units of A per 100 gm. of diet. The cottonseed flour contains 40% of a relatively good quality of protein and also satisfies the B complex requirements.

On account of seasonal, or recurrent aperiodic variations, it is always desirable to conduct all experiments to be compared with each other at the same time. This was obviously

not possible in this extended series. In general, however, it can be said that since a large part of the data covers a planned distribution and no marked seasonal variations happened to enter, the results are sufficiently comparable. In two cases where seasonal variation had to be carefully excluded special seasonal comparisons were made (see table 2 and fig. 3).

TABLE 1

Body weight from fourth to seventh week and per cent bone ash at 7 weeks of age.

DIET	SEX	N	WEIGHTS				PER CENT BONE ASH AT 7 WKS.	GROUP NO.
			Age 4 wks.	5 wks.	6 wks.	7 wks.		
			gm.	*gm.*	*gm.*	*gm.*		
Stock	F	112	45.4±3.8	60.3	75.0	86.9±12.7	58.0±1.4	2
	M	112	49.1±5.7	66.6	84.9	101.1±17.8	57.6±1.5	
Stock + D	F	51	45.4±4.0	61.2	78.1	90.8±15.0	57.9±1.2	1
	M	49	50.6±5.9	68.6	89.0	104.3±20.7	57.3±1.6	
803	F	241	45.4±6.2	52.5	62.3	71.0±10.2	39.3±3.7	5
	M	253	47.8±6.3	56.8	65.1	76.8±11.9	37.0±3.6	
803 + D	F	43	43.8±4.1	49.6	58.4	68.6± 7.8	51.6±1.4	6
	M	49	46.2±5.7	52.2	62.9	72.7±12.8	50.2±1.8	
803 + P	F	39	43.6±2.6	58.8	72.6	83.5± 9.3	58.0±1.0	4
	M	49	46.9±4.8	65.0	81.0	97.4±14.7	56.9±1.3	
803 + P + D	F	25	43.4±3.1	59.7	73.6	85.6± 9.6	59.3±1.0	3
	M	25	47.2±4.8	65.9	80.8	94.5±15.8	58.5±1.1	
2965	F	40	43.3±3.3	49.2	55.5	60.8± 8.8	37.4±3.9	8
	M	56	44.6±5.5	50.3	56.8	63.4± 9.7	36.1±4.6	
2965 + D	F	26	43.4±2.8	48.2	54.5	60.4± 5.0	49.8±1.6	9
	M	40	46.0±4.6	51.4	58.8	64.7± 8.5	49.1±1.8	
2965 + P	F	13	43.6±2.2	50.8	57.6	65.6± 9.6	55.7±1.6	7
	M	7	48.0±3.6	55.6	61.7	67.3±10.6	53.4±2.3	

The ± value given after the mean is the standard deviation, $\sigma = \sqrt{\frac{\Sigma d^2}{N}}$. Group numbers are the identifying numbers used in figures 1 and 2. N = number of animals.

Weekly weighings were made over the 3 week experimental period. At 7 weeks the rats were autopsied. Two criteria of the extent of rickets were used: the percentage of ash in the dry fat-free femur, and the width of the uncalcified cartilage (rachitic metaphysis) of the tibia. The latter has been less extensively employed but if properly measured is of equal significance (Steenbock et al., '25; Dodds and Cameron, '38;

Russell et al., '39). The cartilage width measurements were made on enlarged photographs ($\times$ 3.3) of split silver-stained tibias, and as reported here are 3.3 times natural width. Further details of the technique, as well as a detailed comparison with ash percentages at various levels of rickets production will have to be reserved for another report. The mean values are presented in tables 1 and 2.

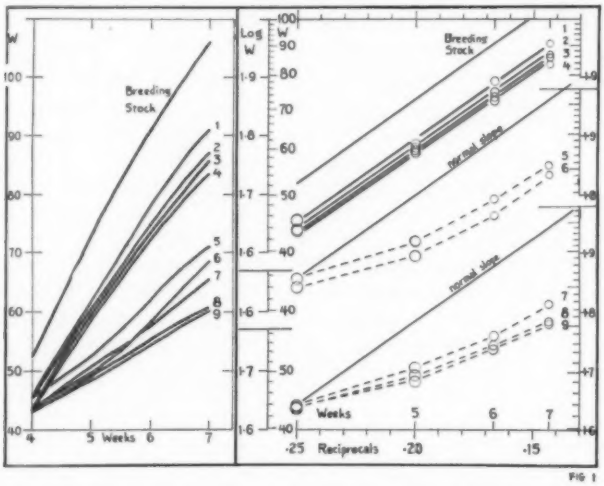


Fig. 1—left hand section Growth of females on the nine rations, plotted as weight against time. 1—stock + D; 2—stock; 3—no. 803 + P + D; no. 803 + P; 5—no. 803; 6—no. 803 + D; 7—no. 2965 + P; 8—no. 2965; 9—no. 2965 + D.

Fig. 1—right-hand section The data of left-hand section replotted as the logarithm of the weight against the reciprocal of time. Each mean weight is represented by a circle of radius 1 gm. In order to avoid crowding the curves have been separated into three groups, each one plotted on a different weight scale (i.e., different origin).

DISCUSSION

Growth. Figure 1 shows the growth of the females on the various rations. The left-hand section of figure 1 shows the data as plotted on ordinary coordinate paper while the right-hand part of the figure makes use of the log-reciprocal plotting

as described in the preceding paper. It can readily be seen that the addition of vitamin D in amounts sufficient to prevent rickets on either rachitogenic diet does not significantly alter the growth during 3 weeks [1] (compare 5 with 6, and 8 with 9, in fig. 1). The phosphate supplement added to no. 2965 (group 7) enhances the growth somewhat and of course prevents rickets. The diet thus supplemented to make it non-rachitogenic, however, does not result in growth as good as that obtained with rickets producing diet no. 803 (group 5). With no. 803 a suitable phosphate supplement (group 4) carries the growth into the range of that recorded for the two stock diet experiments (with D: group 1; without D: group 2).

For a further analysis of these results it is necessary to turn to the log-reciprocal graphs (fig. 1, right-hand section). The growth of rats on diets with no known deficiencies shows a straight line of constant slope when the logarithm of the body weight is plotted against the reciprocal of time (Zucker et al., '41). All deficiencies tried so far result in deviations from this line, which deviations will vary according to the type and degree of the deficiency. The growths on no. 2965 (group 8), no. 2965 plus D (group 9), no. 2965 plus phosphate (group 7), no. 803 (group 5), and no. 803 plus D (group 6) are obviously characteristically deficient. Diets no. 803 plus phosphate (group 4), no. 803 plus phosphate plus D (group 3), and the stock diet (groups 2, 1) each plot quite acceptable straight lines with slopes differing to an insignificant extent from our colony norm. It will be noted that this formulation allows one to compare quality of growth in groups of different inherent size. Under these conditions neither body weight achieved nor gain in weight is a suitable measure.

It can be reasonably concluded that diet no. 803 is deficient only in phosphate, since the addition of adequate phosphate

[1] A well-known fact for diets of the low phosphorus rickets producing type. This must be carefully distinguished from the marked effect on growth of the addition of vitamin D to diets in which there is a relative calcium deficiency. See Zucker, Hall and Young ('38), and Krieger, Bunkfeldt and Steenbock ('40).

(group 4) results in normal growth [2] with rather exacting criteria applied to the term "normal". A further vitamin D supplement (group 3) produces no change in growth just as is the case when it is added to the stock diet.

The intensity of rickets produced on diet no. 803 as compared with diet no. 2965. Table 2 presents data on bone ash per cent and cartilage width measurements obtained from comparable experiments on the two diets. In order to avoid the possible criticism that seasonal variation might play a role we have selected from table 1 rats from experiments which

TABLE 2

Comparison of rickets production on two rachitogenic diets.

DIET	NO. OF ANIMALS		CARTILAGE WIDTH		BONE ASH %	
	♀	♂	♀	♂	♀	♂
			21 day results			
2965	21	26	5.75±0.18	5.42±0.29	39.06±0.92	37.86±1.11
803	32	36	5.70±0.14	6.26±0.17	38.61±0.75	36.13±0.70
			31 day results			
2965	17	20	6.73±0.15	6.95±0.13	35.26±1.03	35.07±0.70
803	16	17	6.63±0.31	6.70±0.32	36.11±1.63	33.51±0.94

The ± figure after the mean value is the standard error $\sigma_m = \frac{\sigma}{\sqrt{N}}$

were conducted at the same time. These animals as indicated above were on the diet for 21 days, and are given in the first section of table 2. The second section deals with similarly treated rats, but the length of the experimental period was 31 days. The difference between the effects of the two diets cannot be considered significant. We have then two diets of the same rickets producing quality as expressed by two separate criteria. There is, however, a difference of some importance; the rickets on no. 803 is uncomplicated rickets since the

[2] While the 3 weeks following weaning cover the most critical period (see Zucker et al., '41), this short time interval hardly constitutes rigorous evidence. Unpublished results have however established that the data obtained with various suitable levels of calcium and phosphorus as supplements to the basal portion of diet no. 803 adhere to the formula for 11 weeks. Incidentally it may be noted that the body weights given by Schneider and Steenbock ('39) on diet no. R14 supplemented with phosphate also give a very acceptable straight line in log-reciprocal plotting.

addition of the single deficient factor (phosphate) produces normal animals both in respect to bone ash per cent and growth. In the case of diet no. 2965 there are other complicating deficiencies besides that of phosphate.

The effects of phosphate and of vitamin D when added to the rickets producing diets. As mentioned above the supplements of phosphate and of vitamin D produced maximal effects as far as rickets prevention is concerned. Larger doses of phosphate tended to the production of low calcium rickets. Further increase in D produced no greater an effect on bone ash or bone picture than that described for the 2 units per day. In each case the cartilage width of the tibias was about 1 mm., which is the value found in stock diet animals of the same age (7 weeks). The 1 mm. in the enlarged photograph is 3.3 times the actual width, and therefore in excellent agreement with the 350 μ actual width recorded by Dodds and Cameron ('38). Microscopic sections also revealed nothing to distinguish such bones from the normal. It is therefore apparent that the supplements here given either to rats on no. 2965 or on no. 803 yielded a bone which was anatomically normal.

In sharp contrast with this uniformity of effect is the widely varying percentage of bone ash (group means 49% to 59%), as well as the considerable range of body weight (group means 60 gm. to 85 gm.).

It is known (see, e.g., Dutcher et al., '25) that the ash per cent in bone increases steadily with age (weight). This is also apparent from the data of Sherman et al. ('25, '31) on per cent of calcium in the body of the rat. Certain generalizations have been made with regard to the relation of parts or components of the body to the body as a whole during the course of growth. Thus Needham ('34) has shown that, using the data of Sherman, the logarithm of the calcium content plots a straight line against the logarithm of the body weight. In view of Needham's reasoning, as well as many other recorded facts, it is advisable to consider body weight in any discussion of the meaning of percentage of ash in the bone.

In figure 2 we have plotted as small points the logarithm of the per cent of bone ash against the logarithm of the body weight of 346 stock diet females varying in body weight from 30 to 150 gm. This gives a picture of the change in bone ash per cent on body weight as well as of the variability encountered in our colony. The heavy-filled circles are the data given in table 1 for females. The lengths of the dotted lines through the points represent the standard deviations on both axes ($\sigma = \sqrt{\frac{\Sigma d^2}{N}}$). The identifying numbers for the points are the same as those used for the curves in figure 1 (in order of

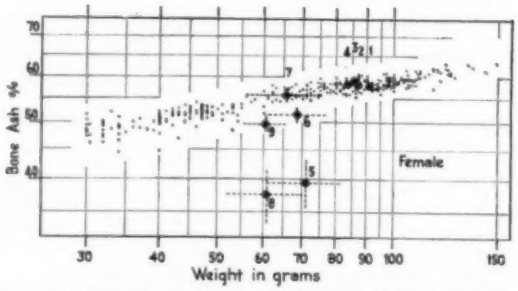


Fig. 2 Log—log plot of bone ash per cent against body weight for females. The small dots represent individual normal stock females, 346 in all, with an age range of 24 to 77 days. It is apparent that this kind of plotting results in a straight line. The large solid circles are the means of table 1 for the females, numbered as before: 1—stock + D; 2—stock; 3—no. 803 + P + D; 4—no. 803 + P; 5—no. 803; 6—no. 803 + D; 7—no. 2965 + P; 8—no. 2965; 9—no. 2965 + D. The lines running through these points represent the standard deviations of the bone ash per cent and the weight. Supplementing either rachitic diet with phosphate results in normal bone ash for the weight achieved; supplementing with D results in bone ashes significantly low even for the weight achieved.

descending final weight). The two stock diet groups (1, 2) as well as the phosphate supplemented groups on no. 803 (3, 4), fall within the range of the small points at body weights normal for rats of the age given (see fig. 1-B). The points for the two unsupplemented rachitogenic diets (5 = no. 803; 8 = no. 2965) fall far below the normal range even for their low body weights. The phosphate supplemented diet no. 2965 (group 7) produces a bone ash percentage not normal for its age, but

precisely normal for the body weight attained. The two remaining points (6 = no. 803 + D; 9 = no. 2965 + D) are the ones of particular interest. The question is: does a vitamin D supplement produce bone ash normal either for age or for body weight? The answer is apparently negative, since they fall with the small standard deviation indicated outside the range of ash for normal stock diet animals. Here again the matter of seasonal effects arises. In figure 3 we show those individual values represented in the mean value of point 6 (no. 803 + D) in figure 2 which could be seasonally balanced with stock diet animals, together with the corresponding males.

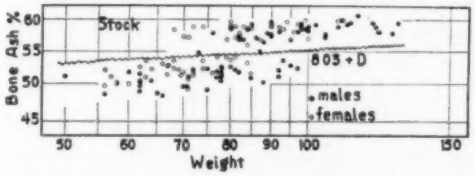


Fig. 3 Log—log plot of bone ash per cent against body weight for males and females. Below the waved line are individual points from the group on no. 803 + D obtained over a short period of time; above the waved line are all the available data on 7-week-old normals on the stock diet covering the same time.

These are all the data points below the waved line. For comparison we have plotted data (male and female) obtained on stock diet animals of the same age, which covered the calendar time of the data on no. 803 + D. Since there is not even any overlapping of individual values, the conclusion seems warranted that diet no. 803 + 2 units of D daily does not result in normal bone ash percentages either for age or for body weight. The same conclusion applies to no. 2965 + D (point 9 in fig. 2). On whatever basis the comparison is made it is apparent that under these conditions—i.e., the usual type of low phosphorus rickets production—optimal doses of vitamin D produce less effect than optimal phosphate supplements.

It is usually stated that vitamin D prevents the rickets of rats on the low phosphorus type of rachitogenic diet. This apparently is not true unless one wants to define rickets on

exclusively morphological grounds. Indications of this were published as early as 1925 (Dutcher et al.). The observation that vitamin D cannot fully replace an absolute phosphate deficiency is hardly worth a lengthy discussion. The interest lies in the fact that some important differences between the usual type of experimental rickets in rats on the one hand, and the spontaneous rickets commonly found in chicks and infants on the other hand, are set out clearly by the conditions here described. In chicks bone ash per cent is brought to normal when under usual conditions vitamin D action prevents the anatomical signs of rickets. In infants calcium and phosphorus retention, and therefore presumably the composition of the bone, are normal under conditions of optimal vitamin D action, while without other changes in the diet, inadequate vitamin D results in rickets. It may, however, even with chicks and infants be quite fallacious to assume that calcium and phosphorus deposition must under all conditions be paralleled by the morphological appearance of the bone, or specifically that normal morphology means normal bone composition. Under the usual conditions of observation infantile rickets and chick rickets can be considered a vitamin D deficiency in the presence of adequate calcium and phosphorus, and the administration of D removes all signs of rickets. Experimental rat rickets is essentially a phosphate deficiency. Only meager attention has been given to the little understood fact that under such conditions vitamin D administration leads to an apparently complete suppression of rachitic bone changes judged visually while in composition the same bone is still quite subnormal, i.e., "rachitic" in the sense of not having one of the chemical criteria of rickets brought into the range of normal. The other chief chemical criterion of rickets, the inorganic blood phosphate, behaves according to Dutcher et al. ('25) in quite the identical manner.

A glance at the values for standard deviation of bone ash reveals a striking difference in variability of normal or near

normal values as against the rachitic ones (see also figs. 2 and 4). This has been previously indicated (Zucker, '36) and is in line with the findings of Massengale and Bills ('36) on chickens. The decrease in variability with very low bone ash values which Bills records does not appear in the rat data here given, possibly because they do not reach the low limiting ash percentage value which, as Bills very logically points out, might again decrease the variability. The statement which we

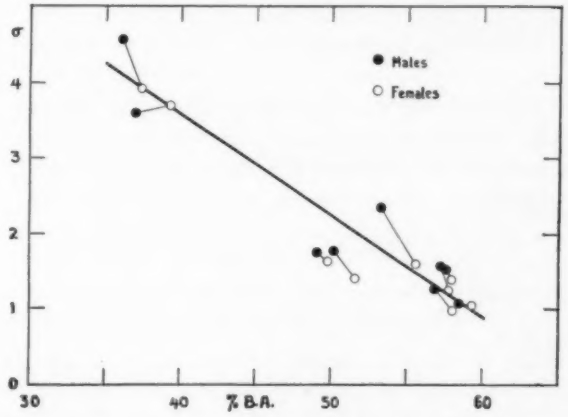


Fig. 4 The standard deviations of the bone ash per cents plotted against the bone ash per cents. The male and female points for each diet are connected. It will be seen that with one exception the male point is to the left of the female point and above it, indicating greater variability and lower bone ash for the males.

(Zucker, '36) have previously made: "It is not surprising to find that the variation in the bone ash values increases the further they are removed from the normal" may have to be amended to take care of the effect of limiting bone ash per cent values. At any rate the data on chicks and on rats are in agreement with respect to the very much larger variability of bone ash per cent values in the rachitic as against the normal range. In quantitative work, as Bills points out, this fact must not be neglected.

SUMMARY

1. A diet comparable in rickets producing quality to the current rachitogenic diets is shown to be adequate for normal growth when phosphate is added.

2. Addition of vitamin D to this or to other low phosphorus rickets producing diets does not lead to normal growth.

3. Addition of vitamin D to rickets producing diets (low phosphorus rickets) prevents the anatomical signs of rickets but does not produce bone ash per cent values equal to those of stock diet animals of the same age or of the same weight; phosphate addition is required to bring the composition to normal.

4. It is pointed out that the visual signs of rickets do not necessarily correspond to bone composition, and the differences between the experimental rickets of rats and the spontaneous rickets in other species are discussed.

LITERATURE CITED

DODDS, G. S., AND H. C. CAMERON 1938 Studies on experimental rickets in rats. II. The healing process in the head of the tibia and other bones. Am. J. Path., vol. 14, p. 273.

DUTCHER, R. A., M. CREIGHTON AND H. A. ROTHROCK 1925 Vitamin studies. XI. Inorganic blood phosphorus and bone ash in rats fed on normal, rachitic and irradiated diets. J. Biol. Chem., vol. 66, p. 401.

HARRIS, R. S., AND J. W. BUNKER 1935 The phosphorus of the corn component of the rachitogenic diet. J. Nutrition, vol. 9, p. 301.

JONES, J. H. 1938 The use of fibrin in synthetic diets. J. Nutrition, vol. 15, p. 269.

KRIEGER, C. H., R. BUNKFELDT AND H. STEENBOCK 1940 Cereals and rickets. X. The availability of phytic acid phosphorus. J. Nutrition, vol. 20, p. 7.

MASSENGALE, O. N., AND C. E. BILLS 1936 A quantitative method for the assay of vitamin D with chickens. J. Nutrition, vol. 12, p. 429.

MCCOLLUM, E. V., N. SIMMONDS, P. G. SHIPLEY AND E. A. PARK 1921 Studies on experimental rickets. VIII. The production of rickets by diets low in phosphorus and fat-soluble A. J. Biol. Chem., vol. 47, p. 507.

NEEDHAM, JOSEPH 1934 Chemical heterogony and the ground-plan of animal growth. Biol. Rev., vol. 9, p. 79.

RUSSELL, W. C., M. W. TAYLOR AND M. T. DUNCAN 1939 Bone development in normal and rachitic rats. J. Nutrition, vol. 18, p. 27.

SCHNEIDER, H. H., AND H. STEENBOCK 1939 A low phosphorus diet and the response of rats to vitamin D. J. Biol. Chem., vol. 128, p. 159.

SHERMAN, H. C., AND A. M. PAPPENHEIMER 1921 Experimental rickets in rats. I. A diet producing rickets in white rats, and its prevention by the addition of an inorganic salt. J. Exp. Med., vol. 34, p. 189.

SHERMAN, H. C., AND F. L. MACLEOD 1925 The calcium content of the body in relation to age, growth and food. J. Biol. Chem., vol. 64, p. 429.

SHERMAN, H. C., AND L. E. BOOHER 1931 The calcium content of the body in relation to that of the food. J. Biol. Chem., vol. 93, p. 93.

STEENBOCK, H., AND A. BLACK 1925 Fat-soluble vitamins. XXIII. The induction of growth promoting and calcifying properties in fats and their unsaponifiable constituents by exposure to light. J. Biol. Chem., vol. 64, p. 263.

STEENBOCK, H., E. B. HART, C. A. ELVEHJEM AND S. W. F. KLETZIEN 1925 Dietary factors influencing calcium assimilation. VI. The antirachitic properties of hays as related to climatic conditions with some observations on the effect of irradiation with ultra-violet light. J. Biol. Chem., vol. 66, p. 425.

ZUCKER, T. F. 1936 Bone ash in prevention and healing of experimental rat rickets. Science, vol. 84, p. 162.

ZUCKER, T. F., L. HALL AND M. YOUNG 1938 Growth promoting effect of vitamin D on low calcium diets. Abstracts, 96th meeting Am. Chem. Soc., Div. of Biol. Chem., p. 17.

ZUCKER, T. F., L. HALL, M. YOUNG AND L. ZUCKER 1941 The growth curve of the albino rat in relation to diet. J. Nutrition, vol. 22, p. 123.

THE EFFECT OF CERTAIN FATS AND UNSATURATED FATTY ACIDS UPON THE UTILIZATION OF CAROTENE [1]

W. C. SHERMAN

Laboratory of Animal Nutrition, Alabama Agricultural Experiment Station, Auburn

TWO FIGURES

(Received for publication January 7, 1941)

Variations in the growth response of rats receiving carotene or vitamin A in different solvents were observed in the early days of vitamin A research. Subsequent investigations indicated that many of the variations in biological response were attributable to differences in the stability of carotene or vitamin A in various natural fats and synthetic esters.

More recently, however, variations in growth response have been reported to occur even though precautions were taken to insure a uniform intake of the active agent. Lathbury and Greenwood ('34) obtained different biological values for carotene when dissolved in arachis oil and coconut oil and in different samples of these solvents. Kraybill and Shrewsbury ('36) found that two to three times as much carotene was required to give equal growth when fed in decolorized butterfat as when fed in cottonseed oil. Basu ('37) testing several oils, obtained the best growth when carotene or vitamin A was fed in linseed oil and the poorest in arachis oil. De ('37) reported that carotene in foods such as amaranth was not as well utilized as carotene in red palm oil.

[1] A preliminary report of this work was presented before the 34th meeting of the American Society of Biological Chemists at New Orleans, La. J. Biol. Chem., vol. 133, p. lxxxix (1940). Published with the permission of the director of the Alabama Agricultural Experiment Station.

In spite of many reports of variations in growth response with various fats it is not possible to correlate the growth effect with any of their known physical or chemical properties. Moreover, there is no evidence of a direct physiological relationship between the metabolism of vitamin A and any other fat constituent.

Although the composition of the basal diets employed by some investigators has not been published, it appears that a relatively low fat content is essential to demonstrate the greatest effect on the growth response to the various oils. However, the possibility of a growth response from the unsaturated fatty acids supplied by certain oils to vitamin A-free diets apparently has not been given due consideration. Burr et al. ('29, '32) demonstrated the essential nature of unsaturated fatty acids and reported that rats receiving a low-fat diet ceased growing soon after the appearance of the early outward signs of an unhealthy condition (seventieth to ninetieth day of life). Hume, Nunn, Smedley-MacLean and Smith ('38) found that the weight curve of rats receiving a low-fat diet began to exhibit a plateau after about 2 months. It was decided, therefore, to feed a low-fat diet and study the effect of various natural oils and unsaturated fatty acids upon the growth response of vitamin A-deficient rats to carotene.

EXPERIMENTAL

Preparation of supplements. Linseed oil, corn oil, wheat germ oil, soybean oil, butterfat, cottonseed oil, and coconut oil were used in feeding. The linseed oil,[2] corn oil,[3] wheat germ oil,[4] and soybean oil [5] were decolorized at room temperature by passage twice through columns packed with fuller's earth. The butterfat was decolorized in a similar manner, but was

[2] H. H. Rosenthal Co., Inc., New York, N. Y.

[3] Mazola brand, Corn Prod. Refining Co., Argo, Ill.

[4] Prepared by ether extraction of "Embo," General Mills, Inc., Minneapolis, Minn.

[5] Prepared by ether extraction of mature soybeans.

maintained at 50°C. during the process. The cottonseed oil [6] and coconut oil [7] were nearly colorless and were not further purified. The methyl linolate was prepared from corn oil and the methyl linolenate from linseed oil according to the methods of Rollett ('09 a, '09 b). The linoleic acid of corn oil was converted into the tetra-bromide and was purified by repeated recrystallizations from skellysolve (Skellysolve B, redistilled) until a beautifully crystalline material was obtained which melted sharply at 114.5° to 114.9°C. The linolenic acid of linseed oil was converted into the hexa-bromide and allowed to precipitate from glacial acetic acid. The hexa-bromide was recrystallized several times from benzene. The final product melted sharply at 179.3° to 179.5°C. Since it is well-known that the free unsaturated fatty acids deteriorate fairly rapidly, the major portion of these acids was stored as tetra- or hexa-bromides. Portions of the bromides were de-brominated every 2 or 3 weeks and esterified for feeding. Representative samples of methyl linolate and methyl linolenate prepared according to this procedure had iodine numbers (Wijs) of 161.0–162.3 and 241.2–242.0, respectively. Skellysolve solutions of methyl linolate and methyl linolenate containing 26.2 gm. of the esters/100 ml. were used in feeding. The usual daily dose of 0.2 ml. of these solutions supplied 0.05 gm. of the free acids. Solutions of carotene [8] in skellysolve containing 15–20 μg./ml. were used in feeding. It has been previously noted (Koehn and Sherman, '40) that solutions of carotene of this concentration are relatively stable; but to insure a constant intake, the exact concentration of carotene in the solutions was determined at bi-weekly intervals by spectrophotometric readings. This method of feeding carotene has an advantage over the usual method of feeding it in various oil solutions in that possible errors resulting from the rapid destruction of carotene in certain oils are completely eliminated. All supplements were stored in glass-stoppered bottles in the dark at 3°C.

[6] Wesson oil.

[7] Pu-re-co brand, Capital City Products Co., Columbus, Ohio.

[8] S.M.A. beta carotene.

Feeding technique. Prior to being placed upon the experimental supplement the rats (Wisconsin strain) were treated in a manner similar to the method used in this laboratory for vitamin A assay (Sherman, '40). When the young rats (usually eight to the litter) reached a weight of 40 to 50 gm. (23 to 26 days) they were transferred to individual cages and fed the vitamin A—test diet 31—A [9] and distilled water ad libitum. No oil supplements were fed during the depletion period. The weekly weights of the rats were recorded during the first 4 weeks of the depletion period, after which the animals were weighed daily until growth had ceased and ophthalmia had developed (usually by the end of the fifth week of depletion). Weekly weights and observations of the gross appearance of the animals were recorded during the remainder of the experiments. In feeding the supplements, aliquots of the skellysolve solutions were pipetted each morning on about 2 gm. quantities of the basal diet contained in separate feed jars. When the skellysolve had evaporated, the contents of the jars were mixed by spatula after which the aliquot of oil (if fed) was added. The jars containing the supplements were immediately placed in the individual cages and the contents were usually consumed within 15 minutes. The animals receiving the various supplements were distributed with respect to sex and litters.

Effect of oils upon the growth response to carotene. The freedom of the oils from vitamin A activity is shown by the results obtained when they were fed without added carotene (table 1). The rats receiving 0.5 ml. of the oils daily continued to lose weight and within 5 weeks all died, exhibiting severe ophthalmia.

The growth of the rats receiving coconut oil and butterfat (0.1 ml. daily) with carotene at levels of 1 and 2 μg. daily was only slightly superior to that of control animals receiving carotene alone. Wheat germ oil, corn oil, linseed oil, and

[9] Diet 31-A was composed of alcohol-extracted casein 18, salt mixture no. 186 (J. Biol. Chem., vol. 89, p. 199, 1930) 4, Northwestern yeast, irradiated 6, and sucrose 72%.

cottonseed oil definitely increased the growth. A still greater increase in growth was obtained with soybean oil at both levels of carotene intake.

Effect of unsaturated fatty acids upon the growth response to carotene. The most obvious difference between the oils which promoted growth and those which did not is the content

TABLE 1

Effect of oils upon the growth response of vitamin A-deficient rats to beta carotene

DAILY SUPPLEMENT		NUMBER OF RATS	MORTALITY	AVERAGE GAIN IN WEIGHT AT END OF				RANGE AT END OF 7TH WEEK
Carotene	Oil [1]			1st week	3rd week	5th week	7th week	
µg.			%	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
..	0.5 ml. coconut oil	2	100	—7	..	..	..	....
..	0.5 ml. wheat germ oil	2	100	—2	—17	..	..	....
..	0.5 ml. cottonseed oil	4	100	—2	—23	..	..	....
..	0.5 ml. linseed oil	4	100	0	—13	..	..	....
..	0.5 ml. soybean oil	3	100	—2	—21	..	..	....
1	.....	14	7	2	7	7	10	–12– 23
1	0.1 ml. coconut oil	4	25	2	5	10	13	3– 25
1	0.1 ml. butterfat	4	0	4	17	23	25	19– 30
1	0.1 ml. linseed oil	9	0	10	29	38	45	25– 80
1	0.1 ml. cottonseed oil	4	0	8	31	43	46	20– 76
1	0.1 ml. soybean oil	4	0	10	36	55	69	42– 92
2	.....	16	6	6	19	25	29	15– 49
2	0.1 ml. coconut oil	6	16	4	15	24	30	13– 41
2	0.1 ml. butterfat	4	25	3	12	25	37	32– 42
2	0.1 ml. wheat germ oil	4	0	10	37	52	62	48– 82
2	0.1 ml. corn oil	3	0	10	34	54	64	55– 73
2	0.1 ml. linseed oil	4	0	8	32	57	70	62– 80
2	0.1 ml. cottonseed oil	6	0	10	40	59	71	49– 85
2	0.1 ml. soybean oil	12	0	13	42	68	91	73–128

[1] All oils except coconut and cottonseed oils were decolorized with fuller's earth.

of essential unsaturated fatty acids. The growth-promoting oils are all known to contain large amounts of linoleic acid; two of them, soybean and linseed oils, contain linolenic acid as well. Butterfat and coconut fat are low in these essential fatty acids. Moreover, the rats which received carotene alone and those which received carotene plus coconut oil or butter-

fat developed a dry scaliness of the skin typical of a deficiency of unsaturated fatty acids. There was definite improvement of the ophthalmic condition of the rats, but a slight swelling of the eyelids usually persisted throughout the entire duration of the experiment with both levels of carotene administration. The skin of the animals receiving the growth-promoting oils was normal in appearance and the ophthalmia was completely cured in nearly all of the rats.

It was decided, therefore, to feed methyl linolate and methyl linolenate to determine if these esters of unsaturated fatty acids would have growth-stimulating properties similar to those of the oils. The results, in the form of growth curves are summarized in figure 1. The growth response obtained when methyl linolate was fed with 2 μg. of carotene was not only inferior to that obtained when the oils were fed with carotene but was invariably inferior to that obtained with carotene alone. The rats gained weight slowly for 2 or 3 weeks, then failed to gain, and finally lost weight throughout the remaining 2 or 3 weeks of the experiments. There was usually a slight improvement in the ophthalmia during the first 2 weeks, then a recurrence, and by the end of the experiments the rats usually exhibited severe ophthalmia. When fed methyl linolenate plus 2 μg. of carotene the animals lost weight, exhibited severe ophthalmia and died before the end of the experiments. Vitamin A, in the form of a skellysolve solution of U.S.P. reference cod liver oil, also was fed with methyl linolate at a level to supply 3.3 U.S.P. XI units of vitamin A, equivalent to 2 μg. of carotene. The methyl linolate had essentially the same antagonistic effect with vitamin A as with carotene, although the animals reached a slightly higher weight before starting to decline.

Because of the excellent growth obtained when carotene was supplemented with soybean oil, it was decided to feed soybean oil with methyl linolate and carotene to determine if it could counteract the antagonistic action of the methyl linolate. The addition of 0.1 ml. of soybean oil to the regular daily carotene

and methyl linolate supplements completely prevented the antagonism (fig. 1, curve E).

With the discovery that methyl linolate impaired the growth of rats on a low level of vitamin A intake, and that this harmful effect could be prevented by soybean oil, it was logical to

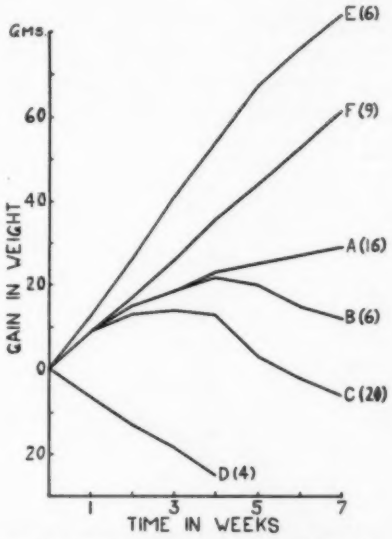


Fig. 1 Average growth curves for rats on low levels of carotene plus unsaturated fatty acids. The numbers in parentheses indicate the number of rats used in obtaining the averages. Curve A, 2 μg. carotene, range at end of experiment 15–49; curve B, 3.3 U.S.P. XI units of vitamin A + 0.05 gm. methyl linolate, range 4–32; curve C, 2 μg. carotene + 0.05 gm. methyl linolate, range —10–18; curve D, 2 μg. carotene + 0.05 gm. methyl linolenate, range —25– —19; curve E, 2 μg. carotene + 0.05 gm. methyl linolate + 0.1 ml. soybean oil, range 53–104; curve F, 2 μg. carotene + 0.05 gm. methyl linolate fed about 6 hours apart, range 40–82.

determine whether impairment of growth was attributable to a chemical or physiological antagonism between the two substances, or to possible toxicity of the methyl linolate with soybean oil merely acting to decrease such toxicity. That the effect was not caused by toxicity of the methyl linolate was shown by experiments in which the methyl linolate and caro-

tene were fed separately a few hours apart. In these experiments, it was the general practice to feed the 2 μg. of carotene early in the morning and the methyl linolate about 6 hours later. From the curve given in figure 1, it is apparent that the antagonism is largely prevented by this method of feeding.

Further evidence for an antagonism between carotene and methyl linolate was obtained by increasing the amount of carotene fed. The growth responses obtained when 5 and 10 μg. of carotene were fed with 0.05 gm. of methyl linolate are shown in figure 2. With 5 μg. of carotene, the methyl linolate

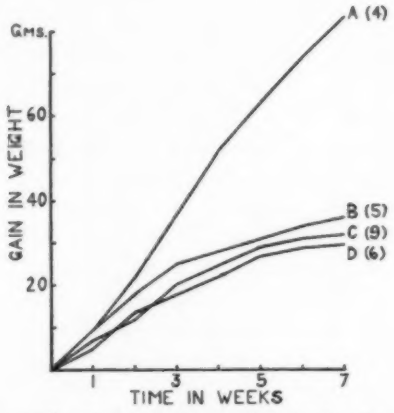


Fig. 2 Average growth curves for rats on high levels of carotene plus methyl linolate. The numbers in parentheses indicate the number of rats used in obtaining the averages. Curve A, 10 μg. carotene + 0.05 gm. methyl linolate, range at end of experiment 58–104; curve B, 10 μg. carotene, range 19–55; curve C, 5 μg. carotene + 0.05 gm. methyl linolate, range 9–68; curve D, 5 μg. carotene, range 15–48.

did not cause the rapid loss of weight which occurred with 2 μg. of carotene; but there was no growth stimulation. There was sufficient antagonism to cause a recurrence of ophthalmia in a number of the rats by the seventh week. When 10 μg. of carotene was fed, the addition of 0.05 gm. of methyl linolate gave a definite growth stimulation and there was no evidence of ophthalmia in the animals.

Effect of methyl linolate upon the stability of carotene. The above results suggested the possibility that the carotene was being destroyed by an oxidation-reduction reaction with the methyl linolate before being consumed by the rats. Evidence of such a reaction has been obtained by Monaghan and Schmitt ('32). To test this possibility, varying amounts of carotene and methyl linolate were mixed with small amounts of the basal diet and exposed to the air in thin layers for periods of 1.5 to 24 hours at 25, 28 and 37°C. The carotene and methyl linolate were then thoroughly extracted with skellysolve. The extract was saponified and analyzed for carotene

TABLE 2

Effect of methyl linolate upon carotene stability

CAROTENE ADDED	ME. LINOLATE ADDED	TIME	TEMPERATURE	CAROTENE RECOVERED	
µg.	*gm.*	*hours*	*°C.*	*µg.*	*%*
25.8	....	3	25	24.2	94.0
25.8	0.20	3	25	24.8	96.2
45.2	....	1.5	28	44.8	99.2
45.2	0.10	1.5	28	45.2	100.0
45.2	....	24	25	30.2	67.0
45.2	0.10	24	25	30.6	67.7
21.8[1]	....	3	37	16.9	77.6
21.8[1]	0.20	3	37	21.7	99.7

[1] In the last experiment the carotene and methyl linolate were incubated with the stomach contents of normal rats.

according to a previously described method (Sherman and Salmon, '39). To determine if gastric acidity were a factor, a test was carried out in which the carotene and methyl linolate were incubated with the gastric contents of normal rats. The results summarized in table 2 show that there was no evidence of direct destruction of the carotene by the methyl linolate; instead, in all instances the methyl linolate apparently exerted a slight protective influence.

Carotene recovered after incubation with methyl linolate was further examined spectrophotometrically and was found to have the same absorption curve as the original carotene. Carotene incubated with methyl linolate was also tested bio-

logically to determine if there were any changes which could not be detected chemically. In this experiment, 636 μg. of carotene and 5.0 gm. of methyl linolate were incubated at 25°C. for 3 hours. The recovered carotene (630 μg.) was then fed to vitamin A-depleted rats at a level of 2 μg. with 0.1 ml. of soybean oil daily. The results given in table 3 show that there was no loss in the biological activity of the carotene by treatment with methyl linolate.

TABLE 3

Biological value of carotene as affected by exposure to methyl linolate

DAILY SUPPLEMENT	NUMBER OF RATS	AVERAGE GAIN IN WEIGHT AT END OF				RANGE AT END OF 7TH WEEK
		1st week	3rd week	5th week	7th week	
		gm.	*gm.*	*gm.*	*gm.*	*gm.*
2 μg. exposed carotene[1] + 0.1 ml. soybean oil	3	15	38	70	86	79–92
2 μg. untreated carotene + 0.1 ml. soybean oil	3	16	42	72	87	71–99

[1] The carotene was allowed to remain in an open jar with methyl linolate for 180 minutes at 25°C.

Effect of oils and methyl linolate upon carotene absorption. Several investigators (Basu, '37; Wilson et al., '36) have attributed variations in the potency of carotene or vitamin A dissolved in various oils to differences in the intestinal absorption of carotene or vitamin A. That mineral oil causes a lowered absorption of carotene is well known. Carotene analyses were therefore made on the feces of rats receiving carotene alone and carotene plus oils or methyl linolate to determine if the difference in the responses obtained could be explained by the effect of the oils upon the absorption of carotene. For these analyses, the fecal material of each rat was collected daily for 7 days and stored at 3°C. under methanol. The feces were then finely ground and analyzed for carotene (Sherman and Salmon, '39). The results from these analyses are summarized in table 4. It is apparent that a relatively efficient absorption of carotene, fed at a level of 5 μg. daily, was obtained regardless of the nature of the oil supplements, al-

though there was slightly more efficient absorption with all of the oils than with the carotene alone. The results, however, do not explain the differences in the growth responses obtained with the various oils and methyl linolate.

TABLE 4

Effect of fats upon carotene absorption by rats

DAILY SUPPLEMENT	NUMBER OF RATS	CAROTENE FED	FECAL CAROTENE [1]	CAROTENE ABSORBED
		μg.	μg.	%
5 μg. carotene	12	35	5.9	83.3
5 μg. carotene + 0.1 ml. coconut fat	8	35	3.9	88.6
5 μg. carotene + 0.1 ml. linseed oil	8	35	4.0	88.5
5 μg. carotene + 0.1 ml. soybean oil	4	35	4.4	87.5
5 μg. carotene + 0.05 gm. methyl linolate	5	35	4.6	87.0

[1] Values for fecal carotene were corrected for the pigment excreted by control rats not receiving carotene (an average of 1.5 μg., calculated as carotene, in 7 days).

DISCUSSION

The results of these experiments demonstrate that with a low-fat diet the response of vitamin A-deficient rats to carotene is influenced by the nature of the oil fed with the carotene. It appears probable that the disagreement between these findings and those of Lease, Lease, Steenbock and Baumann ('39) can be attributed to differences in the fat content of the basal diets used. These workers obtained approximately equal growth responses when carotene was fed with cottonseed oil, soybean oil, lard, decolorized butterfat, coconut oil, or peanut oil at a 3% level to rats which had been depleted of vitamin A on a basal diet containing 10% fat. The growth-stimulating power of the oils seems to be partially dependent upon the content of essential unsaturated fatty acids. However, the feeding of methyl linolate and methyl linolenate with low levels of carotene or vitamin A has revealed the existence of

a very interesting antagonistic relationship. This antagonism is characterized by a retarded growth rate and an incomplete cure of ophthalmia. It appears that the unsaturated fatty acids interfere with some phase of the metabolism of carotene or vitamin A since the harmful effect of the methyl linolate is largely overcome by feeding the two materials a few hours apart, during which time the animals are apparently able to convert either the free linoleic acid or carotene into some less reactive form. That this antagonism does not appear when natural oils high in linoleic acid content are fed is possibly attributable to the presence of some other factor or factors in the oils which counteract this antagonism.

On the basis of these findings, it would appear advisable for investigators who are employing unsaturated fatty acids in feeding to give the vitamin A supplement separately to insure its effective utilization.

SUMMARY

Various natural oils were fed to vitamin A-deficient rats receiving controlled levels of carotene. Of the oils tested, soybean oil gave the best growth. Cottonseed oil, linseed oil, corn oil, and wheat germ oil also had a beneficial effect upon the growth. Butterfat and coconut oil had no appreciable effect.

Tests with methyl linolate and methyl linolenate revealed an antagonistic action when fed with low levels of carotene. When sufficient carotene was fed with the methyl linolate, this antagonism was overcome and the methyl linolate gave a definite growth stimulation. This antagonism was counteracted at the low level of carotene intake by the addition of soybean oil and also by feeding the carotene and methyl linolate a few hours apart.

Carotene analyses of the feces of the rats showed that the differences in the growth response to various oils and methyl linolate could not be explained on the basis of differences in the effect of the oils on the absorption of carotene.

LITERATURE CITED

BASU, N. K. 1937 Vitamin A and fat metabolism. Z. Vitaminforsch., vol. 6, p. 106.

BURR, G. O., AND M. M. BURR 1929 A new deficiency disease produced by the rigid exclusion of fat from the diet. J. Biol. Chem., vol. 82, p. 345.

BURR, G. O., M. M. BURR AND E. S. MILLER 1932 On the fatty acids essential in nutrition. J. Biol. Chem., vol. 97, p. 1.

DE, N. K. 1937 The assimilation of vitamin A and carotene by rats from some common foods with a note on the conversion factor, I.U./E., proposed by the International Vitamin Conference. Indian J. Med. Research, vol. 24, p. 751.

HUME, E. M., L. C. A. NUNN, I. SMEDLEY-MACLEAN AND H. H. SMITH 1938 Studies of the essential unsaturated fatty acids in their relation to the fat-deficiency disease of rats. Biochem. J., vol. 32, p. 2162.

KOEHN, C. J., AND W. C. SHERMAN 1940 The determination of vitamin A and carotene with the photoelectric colorimeter. J. Biol. Chem., vol. 132, p. 527.

KRAYBILL, H. R., AND C. L. SHREWSBURY 1936 The relative vitamin A potency of carotene fed in butter fat and cottonseed oil. J. Nutrition, vol. 11, p. 103.

LATHBURY, K. C., AND G. N. GREENWOOD 1934 The influence of the solvent on the biological effect of carotene and vitamin A. Biochem. J., vol. 28, p. 1665.

LEASE, E. J., J. G. LEASE, H. STEENBOCK AND C. A. BAUMANN 1939 The biological value of carotene in various fats. J. Nutrition, vol. 17, p. 91.

MONAGHAN, B. R., AND F. O. SCHMITT 1932 The effects of carotene and of vitamin A on the oxidation of linoleic acid. J. Biol. Chem., vol. 96, p. 387.

ROLLETT, A. 1909 a Linoleic acid. Z. Physiol. Chem., vol. 62, p. 410.

———— 1909 b Linolenic and linseed oil. Z. Physiol. Chem., vol. 2, p. 422.

SHERMAN, W. C., AND W. D. SALMON 1939 Carotene content of different varieties of green and mature soybeans and cowpeas. Food Research, vol. 4, p. 371.

SHERMAN, W. C. 1940 Chromatograhic identification and biological evaluation of carotene from mature soybeans. Food Research, vol. 5, p. 13.

WILSON, H. E. C., B. AHMAD AND B. N. MAJUMDAR 1936 Further observations on the metabolism of carotene. Indian J. Med. Research, vol. 24, p. 399.

THE VALUE OF UREA IN THE SYNTHESIS OF PROTEIN IN THE PAUNCH OF THE RUMINANT

I. IN MAINTENANCE [1]

LORIN E. HARRIS AND H. H. MITCHELL
Division of Animal Nutrition, University of Illinois, Urbana

TWO FIGURES

(Received for publication February 17, 1941)

In 1911, Armsby reviewed the literature on the subject of the synthesis of protein from non-protein compounds in the alimentary canal of animals, and concluded (1) that there was no evidence that the monogastric animal could utilize the non-protein compounds in this way, but (2) that with polygastric animals the synthesis by the micro-organisms of the paunch is demonstrable but quite limited in extent. The literature was again reviewed by Mitchell and Hamilton in 1929 to essentially the same effect. A third critical review of the literature on the subject was published by Krebs in 1937. While admitting that the evidence indicated some protein sparing effect of such compounds as urea and glycocoll, Krebs was reluctant to believe that any considerable protein synthesis resulted, or that, if it did result, the bacterial protein produced was of any appreciable value to the host.

However, experiments published since the review of Krebs have supplied convincing evidence that urea and ammonium salts may, to a considerable extent, serve the functions of protein in the ruminant. The nitrogen balance experiments of Fingerling and his associates ('37) are clear-cut, while the

[1] The data reported in this paper were taken from a thesis submitted by Lorin E. Harris to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Husbandry, August, 1940.

growth experiments of Bartlett and Cotton ('38), and particularly of Hart, Bohstedt, Deobald and Wegner ('39) furnish good evidence that low-protein rations, inadequate for the growth of calves, are effectively supplemented by urea. And finally attention may be called to the excellent work of Studt ('39) who, using an experimental method analogous to that employed by McElroy and Goss ('39, '40 a, '40 b) in demonstrating vitamin synthesis in the paunch, showed that in sheep subsisting on a low-protein amide-containing ration there is an accumulation of true protein in the paunch 18 hours after the last feeding.

The experiments described below were undertaken with the conviction that a considerable conversion of urea into protein in the paunch has been demonstrated. The main purpose of the experiments was, first, to determine whether the rate of conversion was sufficient to cover the maintenance requirements of sheep, and, second, to measure the efficiency of conversion by determining the biological value of urea in comparison with that of casein.

PLAN OF THE EXPERIMENTS

The experiment consisted of a series of nitrogen balance studies on eight grade sheep, mainly of Rambouillet breeding, ranging from 15 to 18 months in age and from 27 to 40 kg. in body weight. The endogenous urinary nitrogen and the metabolic fecal nitrogen were determined on all sheep by measuring the output of nitrogen in urine and feces after subsistence upon a low-nitrogen ration until the output of urinary nitrogen and the percentage of creatinine nitrogen on the total nitrogen became constant from day to day. Then the nitrogen metabolism was studied in periods of 6 to 34 days in length during which the basal ration was supplemented with graded amounts of urea or casein.

In order to test the finding of Scheunert, Klein and Steuber ('22) that ingested urea is in considerable part excreted through the skin of sheep, all of the sheep in this experiment were washed thoroughly before and after each collection period

with a warm solution of soap and sodium carbonate and rinsed with about 17 liters of distilled water. To facilitate this treatment, the sheep were sheared prior to the initiation of the metabolism experiments. During these experiments the sheep were confined in metabolism crates of a design similar to that described by Forbes ('15).

The low-nitrogen basal ration was modified frequently at the start of the experiments in order to secure a mixture of satisfactory palatability. That used in most of the experiments contained in per cent: wheat straw 25.0, wood pulp 10.5, corn starch 28.42, brown sugar 29.0, corn oil 4.5, complete salt mixture 2.0, and citric acid 0.58. The mixture was dextrinized by steam cooking with water. It was then dried and broken into small lumps. In this form it contained on an average 95.71% of dry matter, 0.136% nitrogen and 4.016 calories of gross energy per gram. In compounding the other rations, urea or casein would be either incorporated in the above ration or, in the case of urea, added to the day's ration. Each sheep received daily, in addition to its solid food, 2 cc. of a fortified cod liver oil.

The food intakes of the sheep were maintained constant insofar as possible throughout a series of comparable metabolism periods, though frequently in a final low-nitrogen period the appetite of the sheep would decline and food consumption would decrease.

All ration mixes, urine collections and feces collections were analyzed for total nitrogen by the Kjeldahl method and the urines were analyzed for creatine and creatinine essentially by the method of Folin ('14) using purified picric acid as described by Benedict ('29) and substituting the photoelectric colorimeter for the DuBosq colorimeter. Cellulose and lignin were determined in food and feces by the methods of Crampton and Maynard ('38).

DISCUSSION OF EXPERIMENTAL RESULTS

The endogenous urinary nitrogen. In twenty metabolism periods on the eight experimental sheep on the low-nitrogen

basal ration, the endogenous nitrogen in the urine averaged 0.0393 gm. daily per kilogram of body weight, or 1.425 gm. per m^2 of body surface (Ritzman and Colovos, '30). Eliminating four high results after the application of Chauvenet's criterion, reduces the average to 0.0333 gm. per kilogram of body weight, or 1.231 gm. per m^2 of body surface daily, the coefficients of variation being 9.43 and 10.40, respectively. The value of 1.231 gm. of endogenous nitrogen per m^2 of body surface is considerably smaller than similar values found by Smuts ('35) for the mouse, rat, guinea pig, rabbit or pig. The value of 0.0333 gm. per kilogram body weight agrees very well with the average of 0.035 gm. for sheep reported by Smuts and Marais ('38), that of 0.037 gm. reported by Miller and Morrison ('39), and also that of 0.0331 gm. obtained by Sotola ('30).

The creatinine nitrogen averaged 24.7% of the total urinary nitrogen in the low-nitrogen periods and the average creatinine coefficient was 22.5. Creatine was present in all of these urines to the extent of 0.032 to 0.244 gm. daily.

The metabolic nitrogen of the feces. In the twenty low-nitrogen periods, the fecal nitrogen excretion averaged 0.555 gm. per 100 gm. of dry matter consumed, with a coefficient of variation of 11.91. This value is identical with that obtained with sheep by Miller and Morrison ('39) and approximates the earlier value of Morgen, Beger and Westhauser (cited by Mitchell, '26), i.e., 0.51 gm. of metabolic nitrogen per 100 gm. of dry matter consumed. It is considerably less than Sotola's ('30) average of 0.66 gm.

The nutritive effects of urea and casein. The appetites of the animals always improved on the transition from the low-nitrogen basal ration to the urea-supplemented or the casein-supplemented rations, and weight maintenance was more readily secured.

The finding of Scheunert, Klein and Steuber ('22) that urea is excreted through the skin of sheep, based upon three washings on one sheep, was not confirmed. The averages collected in table 1 show that the amount of nitrogen contained in the

TABLE 1

Average daily amounts of nitrogen in the wool washings for the different experimental rations

RATION	NUMBER OF WASHINGS	AVERAGE LENGTH OF PERIODS	NITROGEN IN WASHINGS	
			Average per day	AVERAGE PER DAY PER KGM. BODY WEIGHT
		days	*gm.*	*gm.*
Normal	7	44.1	0.133	0.0037
Casein	7	16.3	0.146	0.0039
Urea	19	21.1	0.112	0.0032
N-low	19	12.8	0.114	0.0036

wool washings is unaffected by the type of ration consumed and is low in all cases. It probably represents a rather constant excretion through the skin, together with urine contamination from the floor of the metabolism cage. In the computations of nitrogen balance reported below, the washings nitrogen has been taken into account as a component of the nitrogen excretion.

The fecal nitrogen increased in the transition from a low-nitrogen ration to one containing either casein or urea. The coefficients of apparent and true digestibility for the casein and urea nitrogen are summarized in table 2. The average true digestibility of casein nitrogen, the metabolic nitrogen

TABLE 2

Average digestion coefficients for casein and urea nitrogen

SHEEP NO.	DIGESTIBILITY OF CASEIN NITROGEN			DIGESTIBILITY OF UREA NITROGEN		
	Number of tests	Apparent digestibility	True digestibility	Number of tests	Apparent digestibility	True digestibility
		%	%		%	%
3	1	23.3	82.5	1	24.3	85.6
4	..	...	...	1	23.0	86.0
5	2	38.8	90.6	5	45.9	91.0
6	2	40.4	94.8	4	34.4	81.7
7	..	...	...	4	44.0	89.4
8	..	...	...	4	54.2	93.5
9	..	...	...	1	24.5	99.2
10	2	49.3	79.6	5	51.3	83.0
Average		38.0	86.9		37.7	88.8

of the feces being duly considered, was 86.9%, while that for urea nitrogen was 88.8%. The fact that the nitrogen of a readily soluble compound like urea is not completely digested by the sheep, is consistent with the hypothesis that it has been converted into bacterial protein in the paunch.

The digestibility of dry matter in the basal ration was distinctly improved by the urea supplements (table 3), while the increases observed with the casein supplements can be accounted for only in part by a possible greater digestibility of casein than of the dry matter of the basal ration. Of the dry matter constituents of the basal ration, the cellulose was

TABLE 3

Average digestion coefficients for dry matter and cellulose

SHEEP NO.	DIGESTIBILITY OF DRY MATTER						DIGESTIBILITY OF CELLULOSE			
	Casein ration		Urea ration		N-low ration		Urea ration		N-low ration	
	No. of tests	Digestibility	No. of tests	Digestibility	No. of tests	Digestibility	No. of tests	Digestibility	No. of tests	Digestibility
		%		%		%		%		%
5	2	64.1	5	66.7	3	62.5	2	42.7	2	31.0
6	2	67.8	4	64.8	5	59.0	1	43.9	4	10.0
7	..	...	4	66.7	2	59.8	2	30.0	2	13.2
8	..	...	4	65.2	2	63.3	4	36.5	2	23.8
9	..	...	1	64.8	2	62.8	1	34.9	2	25.0
10	2	68.7	5	66.3	3	59.1	2	44.4	2	3.8
Average		66.9		65.8		61.1		38.7		17.8

digested to a much greater extent (38.7%) in the rations containing urea than in the unsupplemented basal ration (17.8%). The digestibility of lignin was not affected by urea, and data on this point are omitted from table 3. The increased digestion (disappearance) of dry matter and of cellulose contained in the basal ration is consistent with the hypothesis that the addition of urea to such a ration stimulates the proliferation of the paunch flora and enhances carbohydrate fermentation there.

The nitrogen balance, consistently negative on the low-nitrogen regime, was always improved when either casein or urea was incorporated in the ration. Furthermore, it was

always possible with either casein or urea to bring the sheep into nitrogen equilibrium or even to promote a positive balance of nitrogen by using large enough supplements of these substances. These facts were demonstrated with all of the experimental sheep, but the data will not be presented in detail. The relation between the nitrogen intakes of the sheep per kilogram of body weight and the balance of nitrogen expressed on the same basis for all of the sheep for which sufficient data were obtained is depicted graphically in the charts. The relationship is evidently rectilinear, or approximately so, within the range of nitrogen intake that prevailed.

A typical series of nitrogen balances on sheep 7 is given in table 4.

TABLE 4

A series of nitrogen metabolism studies on sheep 7. Results expressed on the daily basis

RATION	LENGTH OF PERIOD	DRY MATTER EATEN	NITROGEN INTAKE		NITROGEN OUTPUT			NITROGEN BALANCE
			In basal ration	In supplement	In feces	In urine	In washings	
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Low-nitrogen	11	371	0.50	0	1.86	0.89	0.07	—2.32
Urea	10	381	0.55	2.32	2.11	1.06	0.06	—0.36
Low-nitrogen	8	302	0.45	0	1.49	0.86	0.07	—1.97
Urea	34	424	0.62	4.63	2.63	1.83	0.06	+0.73

For all sheep, the basal ration provided only from 10 to 30% of the endogenous loss of nitrogen, averaging 22.4%, except in two periods with sheep 5, 6 and 10, when a sample of wheat straw much higher in nitrogen than usual was inadvertently included in the basal ration.

The significance of the nitrogen balances may be questioned on the grounds, either (1) that there is a loss of ammonia nitrogen in the expired air, since urea is rapidly converted to ammonia in the paunch (Lenkeit and Becker, '38), or (2) that there is an accumulation of urea in the blood of ruminants fed this compound. The first possibility was explored with negative results by Scheunert, Klein and Steuber ('22). Wegner, Booth, Bohstedt and Hart ('40) could detect no loss of nitro-

gen from an incubating mixture of rumen bacteria and urea. The absorption of ammonia from the paunch and its excretion through the lungs seems impossible, since the lungs are impermeable to ammonia (Magnus, '02). The second possibility was studied by Bartlett and Cotton ('38) and by Hart and associates ('39) with negative results.

Confirming the significance of the nitrogen balance data relative to the efficacy of urea in covering the nitrogen requirements of maintenance, are the protracted experiments on sheep 7 and 8. The body weights of these sheep were maintained for 109 and 122 days respectively on the low-nitrogen basal ration with additions of urea sufficient to establish a small positive nitrogen balance. At the end of these periods, the health and appetite of the sheep were good and the experiment probably could have been continued indefinitely.

The amounts of urea and casein nitrogen required for equilibrium. The relation between the daily intakes of nitrogen and the daily balances of nitrogen for the various experimental periods and sheep, both quantities expressed per kilogram of body weight, are shown graphically in charts 1 and 2. The data for urea were sufficiently numerous for sheep 5, 6, 7, 8 and 10 to warrant individual presentation. A composite diagram for these sheep, including also the less complete data for sheep 3, 4 and 9, is also presented. The data for casein are composited in one diagram.

The relationship appears to be at least roughly rectilinear and hence in each diagram a rectilinear equation was fitted to the data by the method of least squares. The intersection of this line with the abscissa representing equilibrium locates the intake of nitrogen required for equilibrium. Except for the small amounts of nitrogen in the basal ration, equivalent to about 20 mg. daily per kilogram of body weight, these intakes of nitrogen required for equilibrium represent either urea nitrogen or casein nitrogen. For the five sheep with sufficient data to warrant this statistical treatment, the amounts of urea nitrogen necessary for equilibrium were 215 mg. per kilogram of body weight daily for sheep 5, 248 mg. for sheep 6, 122 mg.

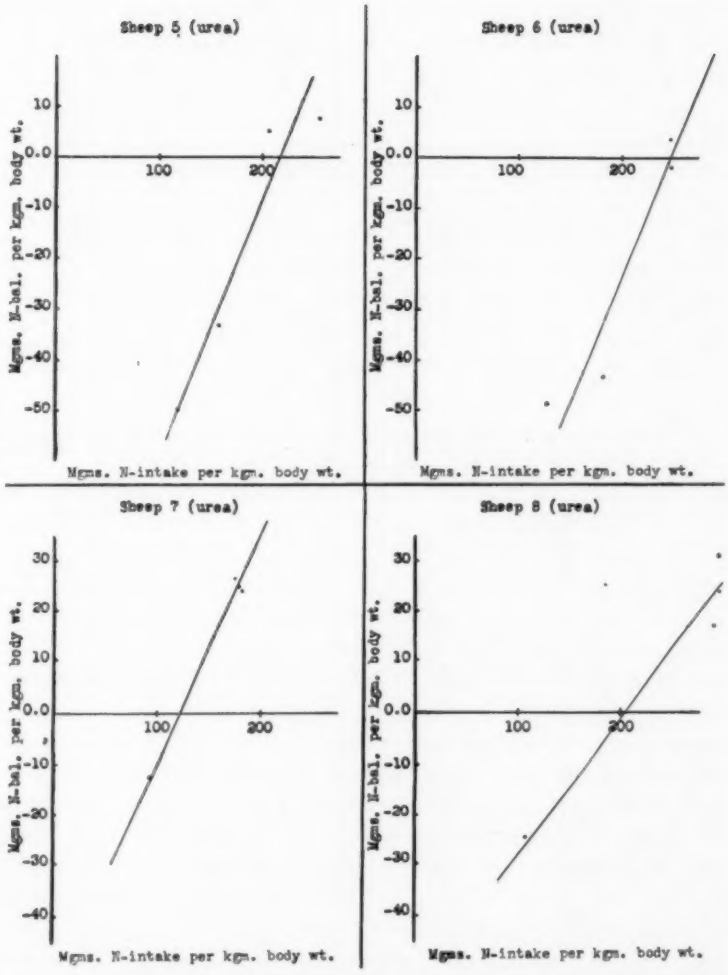


Fig. 1 The relation between the amounts of urea nitrogen and casein nitrogen consumed daily and the nitrogen balance.

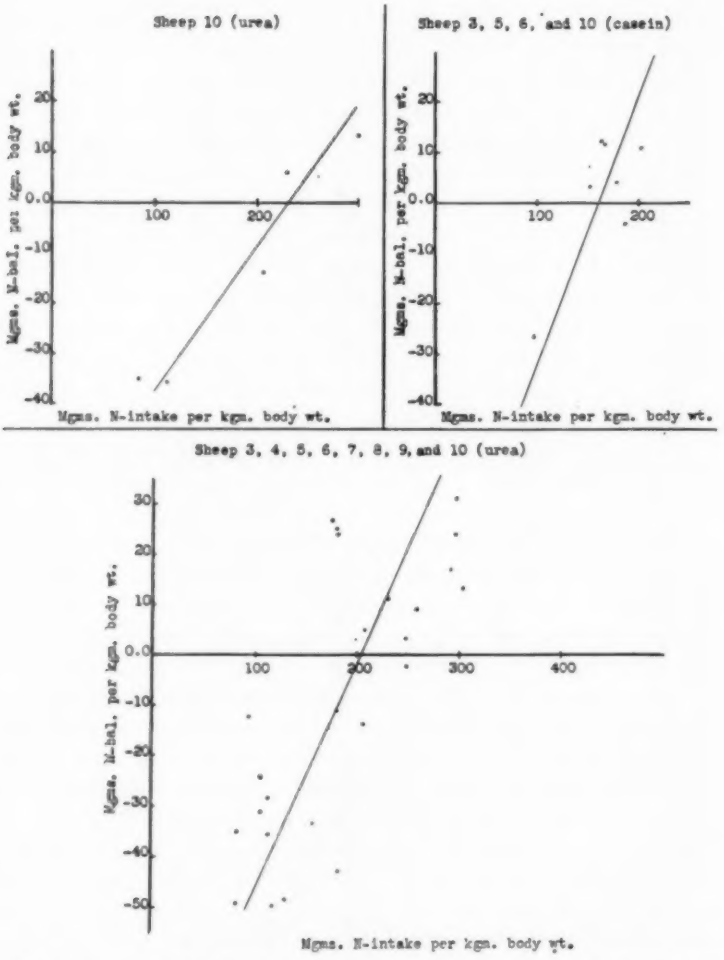


Fig. 2 The relation between the amounts of urea nitrogen and casein nitrogen consumed daily and the nitrogen balance.

for sheep 7, 204 mg. for sheep 8 and 229 mg. for sheep 10. The greater efficiency of sheep 7 in this respect is clearly evident from chart 1. The average of the above values is 204 mg., and the intercept in the composite diagram, containing also less complete data for three other sheep, is 202 mg.

From the composite diagram for the casein experimental periods an intercept of 161 mg. per kilogram of body weight is obtained. A comparison of the two average values, 202 mg. for urea nitrogen and 161 mg. for casein nitrogen, indicates that the former is only 80% as valuable as the latter in the replacement of the losses of endogenous nitrogen in the sheep.

Klein, Schmid, Studt and Müller ('39), from metabolism experiments on five sheep weighing about 50 kg. and subsisting on a ration of molasses, straw, chaff, starch, sucrose, lactose and salt, calculated a physiological protein minimum of 410 mg. of digestible protein per kilogram of body weight necessary for nitrogen equilibrium. This value was obtained by multiplying the urinary nitrogen at equilibrium by 6.25. It relates mainly to the amide nitrogen of molasses which provided most of the nitrogen consumed. In terms of nitrogen, the above value is equivalent to 66 mg.

The values obtained in this study refer to the nitrogen intake, and include not only the indigestible nitrogen of the feces but also the metabolic nitrogen. To obtain values comparable to that of Klein and his associates, they must be multiplied by 0.38, since the apparent digestibility of the dietary nitrogen was 38% (table 2). The product of 202×0.38 is 78 mg., and that of 161×0.38 is 61 mg. The value of Klein et al., i.e., 66 mg., is almost midway between the two values thus computed.

The biological values of urea nitrogen and casein nitrogen for maintenance. Biological values for urea nitrogen and casein nitrogen were calculated in the usual way (Mitchell, '24). The results of these calculations, arranged in the order of increasing levels of dietary nitrogen, are summarized in table 5.

TABLE 5

Estimations of biological values of urea and casein nitrogen

SHEEP NO.	DAILY NITROGEN INTAKE			DAILY NITROGEN BALANCE	BIOLOGICAL VALUE
	Amount	Multiple of N requirement [1]	Protein equivalent in ration [2]		
			Urea		
	gm.		%	gm.	%
4	2.22	0.56	4.13	—1.48	99
10	2.34	0.66	3.40	—1.22	89
3	2.84	0.89	4.23	—0.99	92
Average	2.47	0.70	3.92	—1.23	93
5	3.14	1.00	4.55	—1.57	44
6	3.07	1.00	4.47	—1.42	67
7	2.32	1.00	3.80	—0.37	92
8	2.75	1.00	4.46	—0.77	79
9	2.75	1.00	3.96	—0.86	75
10	3.51	1.00	5.09	—1.29	60
Average	2.92	1.00	4.39	—1.05	70
5	4.19	1.33	6.09	—1.02	43
7	4.63	2.00	6.83	+0.73	76
5	7.44	2.49	8.06	+0.20	60
6	8.37	2.82	9.91	+0.03	54
8	8.25	3.00	11.91	+0.71	49
5	9.30	3.11	10.12	+0.31	49
10	8.33	3.37	9.57	+0.47	42
10	9.59	4.44	12.65	+0.54	36
			Casein		
3	2.43	0.76	4.26	—0.76	100
5	5.31	1.78	5.79	+0.13	87
6	5.31	1.79	6.32	+0.42	88

[1] Multiple of negative nitrogen balance on low-nitrogen ration.

[2] (N × 6.25) expressed as a percentage of the dry matter, disregarding the small amount of nitrogen in the basal ration.

The results show the anticipated drop in the biological value of urea nitrogen with increasing levels of intake. They also reveal for urea an unusually wide range of values for the same level. The exceptionally high biological values obtained consistently for sheep 7 are particularly notable. Since the utilization of urea is a function of the paunch flora and fauna, the variation in utilization would be associated with variations from one animal to another and from one time to another

in the types of micro-organisms present in the paunch and in the conditions existing there bearing on the development and propagation of bacteria and protozoa.

At the lowest levels of intake the urea nitrogen seems to be practically completely utilized in metabolism. At the same concentrations of dietary nitrogen, the nitrogen of casein seems to be definitely better utilized than that of urea.

It is possible to compute the biological values of the nitrogen of urea and casein at the precise point of nitrogen equilibrium from the data given in the last section relative to the amounts of urea and casein nitrogen, respectively, required for equilibrium, i.e., 202 and 161 mg. per day per kilogram of body weight. This computation is made according to the following formula:

$$B = \frac{E}{ID - \frac{M}{B}}$$

in which B is the biological value expressed as a decimal, E is the endogenous nitrogen, I the nitrogen intake required for equilibrium, M the metabolic nitrogen, and D the true digestibility of the dietary N, expressed as a decimal. Per kilogram of body weight per day, $E = 33.3$ mg. and $M = 77.4$ mg., $I = 202$ mg. for urea nitrogen and 161 mg. for casein nitrogen and $D = 0.888$ for urea nitrogen and 0.869 for casein.

Thus computed, the average biological values of urea and casein nitrogen at nitrogen equilibrium are 62 and 79, respectively.

CONCLUSIONS

1. The endogenous nitrogen excretion of sheep weighing about 34 kg. and ranging in age from 15 to 18 months averages 0.0333 gm. per kilogram of body weight and 1.23 gm. per m^2 of body surface. The metabolic nitrogen in the feces averages 0.55 gm. per 100 gm. of dry matter consumed.

2. Urea administered to sheep in amounts commensurate with their requirements for maintenance is not excreted through the skin.

3. Urea added to a low-nitrogen ration improves the digestibility of cellulose markedly and is itself digested to the extent of 88.8%.

4. Sheep may be maintained in body and nitrogen equilibrium for well over 100 days on rations containing urea and minimal amounts of protein providing only one-tenth the amount of nitrogen needed for equilibrium.

5. Nitrogen equilibrium may be maintained on 202 mg. of urea nitrogen and 161 mg. of casein nitrogen per kilogram of body weight daily.

6. At nitrogen equilibrium, the biological value of urea nitrogen is 62, and of casein nitrogen 79.

The authors wish to acknowledge the invaluable advice and assistance of Dr. W. G. Kammlade and Mr. W. J. Hampton of the Sheep Husbandry Division in the feeding and care of the sheep used in these experiments.

LITERATURE CITED

ARMSBY, H. P. 1911 The nutritive value of the nonprotein of feeding stuffs. U. S. Dept. Agr., Bur. An. Ind., Bul. 139, p. 49.

BARTLETT, S., AND A. G. COTTON 1938 Urea as a protein substitute in the diet of young cattle. J. Dairy Res., vol. 9, pp. 263–272.

BENEDICT, S. R. 1929 A note on the purification of picric acid for creatinine determination. J. Biol. Chem., vol. 82, pp. 1–3.

CRAMPTON, E. W., AND L. A. MAYNARD 1938 The relation of cellulose and lignin content to the nutritive value of animal feeds. J. Nutrition, vol. 15, pp. 383–396.

FINGERLING, G., B. HIENTZSCH, H. KUNZE AND K. REIFGERST 1937 Ersatz des Nahrungeiweisses durch Harnstoff beim wachsenden Rinde. Landw. Vers.-Stat., vol. 128, pp. 221–235.

FOLIN, O. 1914 On the determination of creatinine and creatine in the urine. J. Biol. Chem., vol. 17, pp. 469–473.

FORBES, E. B. 1915 A metabolism crate for swine. Ohio Agr. Exp. Sta. Circ. 152, pp. 75–85.

HART, E. B., G. BOHSTEDT, H. J. DEOBALD AND M. I. WEGNER 1939 The utilization of simple nitrogenous compounds such as urea and ammonium bicarbonate by growing calves. J. Dairy Sci., vol. 22, pp. 785–798.

KLEIN, W., H. SCHMID, E. STUDT AND R. MÜLLER 1939 Der Strukturwert des aus nichteiweissbartigen Verbindungen im Pansen erzeugten Bakterieneiweisses auf Grund des festgestellten physiologischen Eiweissminimums. Z. Tierzücht. u. Züchtungsbiol., vol. 43, pp. 76–110.

KREBS, K. 1937 Der Wert der Amide bei der Fütterung des Rindes. Historische Betrachtung der Entwicklung der Amidfrage, Kritische Wertung des Standes unserer heutigen Kenntnisse. Biedermann's Zentralbl. Agrikulturchem. Abt. B. Tierernähr., vol. 9, pp. 394–507.

LENKEIT, W., AND M. BECKER 1938 Das Schicksal des Harnstoffs der Amidflocken im Pansen. Zeit. Tierernähr. in Futtermittelkunde, vol. 1, pp. 97–101.

MAGNUS, E. 1902 Ueber die Undurchgängigkeit der Lunge für Ammoniak. Arch. exp. Pathol. u. Pharm., vol. 48, pp. 100–106.

MCELROY, L. W., AND H. GOSS 1939 Report on four members of the vitamin B complex synthesized in the rumen of the sheep. J. Biol. Chem., vol. 130, pp. 437–438.

———— 1940 a A quantitative study of vitamins in the rumen contents of sheep and cows fed vitamin-low diets. I. Riboflavin and vitamin K. J. Nutrition, vol. 20, pp. 527–540.

———— 1940 b A quantitative study of vitamins in the rumen contents of sheep and cows fed vitamin-low diets. II. Vitamin B_6 (pyridoxine). J. Nutrition, vol. 20, pp. 541–550.

MILLER, J. I., AND F. B. MORRISON 1939 Relative efficiency for growing lambs of the protein in rations containing alfalfa hay, timothy hay, and combinations of the two hays. J. Agr. Res., vol. 58, pp. 149–155.

MITCHELL, H. H. 1924 A method of determining the biological value of protein. J. Biol. Chem., vol. 58, pp. 873–903.

———— 1926 The determination of the protein requirements of animals and of the protein values of farm feeds and rations. Bull. Natl. Res. Council no. 55, 44 pp.

MITCHELL, H. H., AND T. S. HAMILTON 1929 The biochemistry of the amino acids. Am. Chem. Soc. Monograph, New York, 619 pp.

RITZMAN, E. G., AND N. F. COLOVOS 1930 Surface areas of sheep. N. H. Agr. Exp. Sta. Circ. no. 30, p. 8.

SCHEUNERT, A., W. KLEIN AND M. STEUBER 1922 Über die Verwertbarkeit des Harnstoffs als Eiweissquelle für Wiederkäuer, zugleich ein Beitrag zur Frage der exkretorischen Funktionen der Haut. Biochem. Z., vol. 133, pp. 137–191.

SMUTS, D. B. 1935 The relation between the basal metabolism and the endogenous nitrogen metabolism, with particular reference to the estimation of the maintenance requirement of protein. J. Nutrition, vol. 9, pp. 403–433.

SMUTS, D. B., AND J. S. C. MARAIS 1938 The endogenous nitrogen metabolism of sheep with special reference to the maintenance requirement of protein. Onderstepoort J. Vet. Sci. and An. Indust., vol. 11, pp. 131–139.

SOTOLA, J. 1930 Biological values and supplementary relations of the proteins in alfalfa hay and in corn and sunflower silage. J. Agr. Res., vol. 40, pp. 79–96.

STUDT, E. 1939 Über die Menge und Verdaulichkeit des durch zymogene Symbiose im Pansen des Wiederkauers erzeugten Bakterieneiweisses auf Grund von Schlachtergebnissen. Z. f. Tierzüch. u. Züchtungsbiol., vol. 44, pp. 253–261.

WEGNER, M. I., A. N. BOOTH, G. BOHSTEDT AND E. B. HART 1940 The "in vitro" conversion of inorganic nitrogen to protein by micro-organisms from the cow's rumen. J. Dairy Sci., vol. 23, pp. 1123–1129.

THE VALUE OF UREA IN THE SYNTHESIS OF PROTEIN IN THE PAUNCH OF THE RUMINANT

II. IN GROWTH [1]

LORIN E. HARRIS AND H. H. MITCHELL
Division of Animal Nutrition, University of Illinois, Urbana

(Received for publication February 17, 1941)

In the preceding paper (Harris and Mitchell, '41) it has been shown that urea nitrogen is available to the sheep for the replacement of at least 90% of the endogenous losses of nitrogen, presumably through the mediation of the micro-organisms of the paunch, with an efficiency of about 60%. In the experiment to be reported below, the extent to which urea nitrogen can be used in satisfying the growth requirements of sheep was explored and some information was obtained on the efficiency of utilization.

PLAN OF THE EXPERIMENT

Twenty-three Montana grade cross-bred wether lambs, ranging in weight initially from 50 to 80 pounds, were the subjects of the experiment. During a preliminary period of 40 days they were fed a ration of corn silage ad libitum, supplemented with limestone, salt and a fortified cod liver oil. The purpose of this period was to determine whether this ration, to be used as a basal ration in the subsequent growth period, was capable of supporting growth or nitrogen equilibrium. Hence, body

[1] The data reported in this paper were taken from a thesis submitted by Lorin E. Harris to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Husbandry, August, 1940.

weights were determined for all lambs at the beginning and end of the period and nitrogen balances measured on three of the lambs.

At the end of the preliminary period, all lambs were sheared and five of them were slaughtered for carcass analysis.

The remaining eighteen lambs were then carried through a growth period of 71 to 111 days. During this period the lambs were fed in six pairs and two trios, for the purpose of comparing the basal ration plus a nitrogen-free supplement (dextrinized by steam cooking) of starch 83, corn syrup 10, brown sugar 5, salt mixture 1 [2] and citric acid 1%, with three rations prepared from the latter by the addition of sufficient urea to raise the protein equivalent (N $\times$ 6.25) from an average of 5.35% on the dry basis to approximately 8% in one case, 11% in a second case and 15% in a third.

Three pairs of lambs were used in comparing the 11 and 15% rations, and three other pairs in comparing the 8 and 11% rations. In one trio, the basal ration was compared with the 8 and 11% rations, and in another trio, the basal ration was compared with the 11 and 15% rations. Within all pairs and trios the intakes of silage and of carbohydrate supplements were equalized.

After the first 30 days of feeding, the Ca to P ratio of all rations was adjusted to approximately 2:1, and elemental sulfur was added to all rations in the proportion of one-fifteenth of the urea nitrogen in the 15% protein-equivalent ration.

Nitrogen balances were determined on the lambs during the growth period, using collection periods of 10 days preceded by preliminary periods of 5 days, the food intake being constant for the entire 15 days. At the termination of the growth period, the lambs were slaughtered and their entire carcasses were analyzed for dry matter, nitrogen and gross energy. Initial and final body weights of the lambs were averages of weights taken on 3 consecutive days.

[2] L. G. Wesson, Science, vol. 75, p. 339 (1932).

THE EXPERIMENTAL RESULTS

Preliminary period. During the 40-day preliminary period the ration of corn silage supplemented by minerals and cod liver oil, in the amounts voluntarily consumed, did not maintain the body weight of most of the twenty-three lambs. With the exception of two of the lambs, the food consumption declined during this period. Two of the lambs showed slight increments in body weight of 1.5 and 3.0 pounds, one lamb maintained its weight while all of the rest lost weight, the losses ranging from 0.5 to 10.0 pounds. The average change in weight for the group was −3.1 pounds.

The nitrogen metabolism was studied on this ration with three of the lambs. The results of these studies are summarized in table 1. During the first collection periods the first

TABLE 1

Nitrogen metabolism of lambs during the preliminary period on the basal ration alone and with a sugar supplement.

LAMB NO. AND BODY WEIGHT	RATION	DAILY NITROGEN METABOLISM:			
		Intake	Feces	Urine	Balance
		gm.	*gm.*	*gm.*	*gm.*
2706	Basal	4.99	2.61	2.98	−0.60
60.3 kg.	Basal + sugar	4.11	3.05	1.67	−0.61
	Basal	4.42	2.65	2.71	−0.94
2837	Basal	4.99	2.40	3.01	−0.42
68.0 kg.	Basal + sugar	4.11	3.24	1.84	−0.97
	Basal	4.88	2.61	2.58	−0.31
2783	Basal + sugar	4.24	4.09	1.44	−1.29
64.5 kg.	Basal	4.42	3.56	1.94	−1.08

two lambs consumed 1361 gm. of silage daily. The nitrogen balances were all negative. In order to determine whether these losses of nitrogen were conditioned by an inadequate intake of energy, 125 gm. of glucose were added to the ration of lambs 2706 and 2837 in a second period and in the first collection period for lamb 2783. The intake of silage was somewhat depressed, amounting to 1225 gm. daily for the first two lambs and 1261 gm. for the third, but the energy intake must nevertheless have been greater. The effect of the sugar was to depress the excretion of urinary nitrogen but to increase that of the fecal nitrogen to such an extent that the balances of nitrogen for the first two lambs were even more

negative than in the first collection period. A return to the silage ration in a following collection period reversed these changes.

The digestibility of the nitrogen of the silage was apparently depressed by the sugar supplement even more than would be expected from the resulting increase in the excretion of metabolic nitrogen in the feces. Computations of the biological value of the nitrogen of the silage, using average values, obtained in the preceding studies on maintenance, for the endogenous nitrogen in the urine, per 100 gm. of body weight, and the metabolic nitrogen in the feces per gram of dry matter consumed, gave an average of 59, with individual values of 56, 52, 59, 63 and 64. For the silage and sugar ration, the biological values of the nitrogen were 73, 76 and 79, averaging 76.

The five check lambs slaughtered at the termination of the preliminary period showed an average gastrointestinal fill of 16.6% of the live weight. The average composition of the empty weight was: 35.53 $\pm$ 0.64% of dry matter, 2.42 $\pm$ 0.05% of nitrogen and 2194 $\pm$ 55 gm. cals. per gram. These average values were used in computing the initial composition of the lambs in the growth experiment.

Growth period. With the addition of the carbohydrate supplement and the urea at the start of the growth period, the lambs ate their rations with more relish and the food consumption increased. The period started on December 12, 1937, and, as in the preliminary period, the lambs were housed at the sheep barns. However, on December 27 a spell of sub-zero weather set in and it was impossible to keep the lambs comfortably warm, even with the use of blankets. They were therefore moved to the nutrition laboratory and confined in individual stalls, improvised from wooden hinged barriers, on a concrete floor covered with shavings. During this disturbance the lambs went off feed and their appetites were slow in recovering under laboratory conditions. Hence, for a period of 20 to 40 days the lambs first lost weight and then merely regained their initial weights. From then on, however, the gains were as satisfactory as could be expected from the nature of the rations under test.

In the course of 50 days it became apparent that the lambs in the two trio groups receiving the basal ration alone were not going to gain in weight. Hence, the control lamb in the trio affording a comparison of the 11 and 15% protein rations was given sufficient casein to bring the protein level to 15%, the starch in the carbohydrate supplement being reduced accordingly. Two balance periods were obtained on this trio after this change in feeding was effected.

The control lamb in the other trio also began to refuse feed before a nitrogen balance study could be made. In this case, all three lambs were put upon a ration of alfalfa hay, corn, oats, yeast and mineral supplements for a period of 20 days, after which they were returned to their original rations and management. On the latter regime, their food consumption was such that a nitrogen balance study was carried out, but shortly after this the appetite of the control lamb again declined.

The average daily gains in body weight of these two trios of lambs during the various subperiods into which their experiments were necessarily divided, are collected in table 2. From

TABLE 2

Summary by periods of daily gains of lambs in trios 1 and 2.

TRIO	LAMB NO.	LENGTH OF PERIOD	RATION FED	PROTEIN EQUIVALENT IN RATION	AVERAGE DAILY GAIN
		days		%	*pounds*
1	2885	50	Basal + urea	14.7	—0.08
	2706	50	Basal + urea	10.9	—0.02
	2850	50	Basal	5.1	—0.19
1	2885	61	Basal + urea	14.2	0.34
	2706	61	Basal + urea	10.6	0.34
	2850	61	Basal + casein	14.4	0.42
2	2754	50	Basal + urea	11.2	—0.11
	2796	50	Basal + urea	8.2	—0.22
	2805	50	Basal	5.8	—0.23
2	2754	20	Normal	16.1	0.65
	2796	20	Normal	16.1	0.78
	2805	20	Normal	16.1	0.75
2	2754	41	Basal + urea	11.2	0.17
	2796	41	Basal + urea	8.2	0.09
	2805	41	Basal	5.8	0.05

these results it is evident, first, that the basal ration alone was unable to support any considerable growth in the amounts consumed, or even to maintain weight, and, second, that the basal ration supplemented with urea was capable of promoting approximately normal gains in body weight.

In confirmation of the latter statement, the three pairs of lambs receiving the rations containing 11 and 15% of protein equivalent made gains during the last 60 days of the experiment averaging 0.26 and 0.25 pound daily, respectively.

The total gains in body weight for the entire growth period (see table 3) reveal a consistent advantage for the 11% ration over the 8% ration among the four possible comparisons. The average difference in total gain was 2.12 kg., the standard deviation of differences, 1.01 kg., and the probability that fortuitous factors would produce an average difference in gain as great or greater than 2.12 kg. is only 0.018 (Student, '08) and hence may be neglected. It may, therefore, be concluded that the additional urea in the 11% ration has induced a greater rate of growth than the 8% ration.

In the four comparisons of the 11 and 15% rations, the average total gains were 8.90 and 7.62 kg., respectively, and the difference between the two averages is clearly insignificant.

The nitrogen metabolism data secured on the lambs during the growth period are summarized in table 4. The three possible comparisons of the rations containing 11 and 8% protein equivalent all favor the higher protein ration in the daily storage of nitrogen, the average difference in nitrogen balance being 1.25 gm. per day. The standard deviation of differences is 0.35 gm. and the probability of a fortuitous result is only 0.018. The nitrogen balance data, therefore, bear out the body weight measurements in indicating a superior performance of the 11% ration over the 8% ration in promoting growth.

The unsupplemented basal ration induced negative nitrogen balances in three of the four tests, and the one positive balance was practically the equivalent of equilibrium, i.e., +0.30 gm. daily.

TABLE 3

The total gains in body weight and the total storage of nitrogen and energy for the growing lambs.

LAMB NO.	PROTEIN EQUIVALENT OF RATION	LENGTH OF FEEDING PERIOD	INITIAL BODY WEIGHT	FINAL BODY WEIGHT	GAIN IN BODY WEIGHT		N CONSUMED		TOTAL STORAGE			BIOLOGICAL VALUE OF TOTAL N
					Total	Average per day	Total	As urea	Dry matter	Nitrogen	Energy	
	%	*days*	*kg.*	*kg.*	*kg.*	*pounds*	*gm.*	%	*gm.*	*gm.*	*therms*	%
2909	11	102	28.6	34.0	5.4	0.117	1037	50.4	2762	147.5	22.86	58
2837	8	102	28.6	33.6	5.0	0.107	762	32.5	1977	86.0	18.90	70
2783	11	71	26.3	25.6	—0.7	—0.021	592	53.5	271	39.9	—0.17	52
2776	8	71	26.1	22.9	—3.2	—0.098	406	36.6	448	41.6	2.19	74
2711	11	71	25.2	30.6	5.4	0.168	835	52.7	2080	110.3	16.02	57
2692	8	71	24.3	26.9	2.6	0.081	614	36.5	1071	93.4	6.73	74
2754	11	111	21.8	28.3	6.5	0.130	768	48.1	1651	173.3	10.19	66
2796	8	111	22.5	26.2	3.7	0.074	573	29.1	1235	86.3	6.69	77
2805	5	111	22.7	25.2	2.5	0.049	418	0	65	47.8	—2.08	100
2773	15	110	33.1	41.4	8.3	0.165	2196	64.2	2479	172.8	17.76	39
2824	11	110	34.0	44.4	10.4	0.202	1631	51.9	4292	243.5	32.69	57
2830	15	110	24.7	34.2	9.5	0.190	1704	58.4	4331	197.6	31.62	42
2629	11	110	24.7	33.7	9.0	0.179	1257	50.0	4018	214.1	25.68	58
2800	15	111	22.7	27.8	5.1	0.101	1317	60.4	2175	70.1	19.49	37
2701	11	111	21.3	28.6	7.3	0.143	969	46.1	1460	122.7	9.16	58
2885	15	111	25.6	33.2	7.6	0.151	1548	63.8	1965	199.1	12.57	46
2706	11	111	25.4	34.3	8.9	0.177	1149	51.3	3366	197.2	24.05	59

TABLE 4

Nitrogen metabolism data on growing lambs. Results expressed on the daily basis.

LAMB NO.	PER CENT PROTEIN EQUIVALENT	NITROGEN SUPPLEMENT	NITROGEN INTAKE		N IN FECES	N IN URINE	N BALANCE	BIOLOGICAL VALUE OF TOTAL N
			Basal ration	Supplement				
			gm.	*gm.*	*gm.*	*gm.*	*gm.*	%
2909	11	Urea	4.90	5.53	3.58	4.10	2.75	67
2837	8	Urea	4.90	2.64	3.35	2.78	1.41	74
2711	11	Urea	6.02	7.51	5.21	4.61	3.71	70
2692	8	Urea	6.02	3.85	5.13	2.65	2.09	79
2754	11	Urea	4.89	4.05	3.18	4.23	1.53	60
2796	8	Urea	4.89	1.72	3.59	2.27	0.75	73
2805	5	None	4.89	0	4.06	1.34	—0.51	63
2805	5	None	4.46	0	3.38	1.48	—0.40	75
2773	15	Urea	5.75	12.39	4.95	11.30	1.89	40
2824	11	Urea	5.75	7.51	4.47	6.26	2.53	60
2830	15	Urea	4.90	9.38	3.21	8.34	2.73	47
2629	11	Urea	4.90	5.53	3.46	4.68	2.29	62
2800	15	Urea	4.92	7.22	2.99	7.61	1.54	41
2701	11	Urea	4.92	3.95	3.13	4.87	0.87	50
2885	15	Urea	4.79	9.12	3.07	7.55	3.29	51
2706	11	Urea	4.79	5.49	3.69	5.30	1.29	53
2850	5	None	4.79	0	3.64	1.45	—0.30	82
2885	15	Urea	5.05	9.38	3.65	8.17	2.61	47
2706	11	Urea	5.05	5.53	3.39	5.05	2.14	59
2850	5	None	5.05	0	3.24	1.51	0.30	83
2885	15	Urea	5.11	8.94	3.98	7.40	2.67	51
2706	11	Urea	5.11	5.25	3.32	4.17	2.87	68
2850	15	Casein	5.07	8.98	3.48	6.80	3.77	57
2885	15	Urea	6.36	9.71	4.39	8.20	3.50	53
2706	11	Urea	6.36	5.60	3.65	5.16	3.15	65
2850	15	Casein	6.32	9.75	4.65	6.93	4.49	61

Comparing the 15 and 11% rations, only five of the seven pairings favor the former ration. The average daily balances of nitrogen were, respectively, 2.60 and 2.16 gm. daily, and the average difference, 0.44 gm., is statistically insignificant ($s = 0.76$ and $P = 0.11$). Thus, again the balance data support the growth data, in this case indicating no significant difference in growth-promoting properties between the 15 and the 11% rations.

The conclusion may, therefore, be drawn from the latter comparison, either that the ration providing 11% protein equivalent satisfies completely the nitrogen requirement of these lambs for maintenance and growth, or that the upper limit in the rate of protein synthesis from urea in the paunch was reached on the 11% ration. The former conclusion is not out of harmony with unpublished experimental results from this laboratory indicating that the growth requirements of lambs of approximately the size of those used in this experiment can be completely satisfied by a ration containing not much more than 9% of protein (Mitchell and Hamilton, '31–'32, '35–'36).

The two tests with casein indicate a clear superiority of casein nitrogen at the level of 15% protein equivalent over the urea nitrogen at either the 15 or the 11% protein equivalent level. If these differences were statistically significant, the conclusion would be justified that the rate of protein synthesis from urea in the paunch is not sufficiently rapid under the conditions of this experiment to provide fully for the protein demands for growth.

It is interesting to note that the digestibility of dry matter was distinctly less for the lambs on the basal ration than for the lambs on the urea-containing and casein-containing rations, averaging 62.2, 73.3 and 71.3%, respectively.

The storages of nitrogen and energy throughout the growth period of all lambs slaughtered and analyzed, estimated from the chemical composition of the entire carcasses and from the average composition of the check lambs sacrificed and analyzed at the beginning of the experiment, are summarized in table 3. Lamb 2850 was not slaughtered, because the change from the basal ration to a casein-containing ration would render ambiguous the results of a carcass analysis. Lamb 2837 died of acute indigestion about 10 days before it was planned to terminate the feeding period, an event bearing no apparent relation to the type of ration consumed. Its pair mate, 2909, was slaughtered at this time and both carcasses were analyzed. Because of the poor food consumption of lambs 2776 and 2692,

both receiving the 8% ration, the feeding period of the corresponding pairs was terminated earlier than planned.

The average compositions of the lambs in the various groups are summarized in table 5.

TABLE 5

The average chemical composition of the lambs on the various experimental rations.

APPROXIMATE PROTEIN EQUIVALENT OF RATION	NUMBER OF LAMBS IN GROUP	COMPOSITION OF EMPTY CARCASSES		
		Dry matter	Nitrogen	Gross energy
%		%	%	*cal. per gm.*
11	4	37.55	2.59	2378
8	4	37.98	2.62	2419
5.8	1	35.41	2.64	2057
15	4	38.26	2.50	2494
11	4	38.50	2.53	2459

These values reveal nothing abnormal in the composition of the carcasses of the lambs receiving the urea rations, and no marked differences in composition produced by the different levels of urea fed.

Among the four possible comparisons of the storage of nitrogen on the 11 and 8% rations, three indicated greater storages on the 11% ration. The one exception relates to the pair of lambs, nos. 2783 and 2776, that had the poorest appetites throughout the growth period and failed even to maintain their initial weight. These conditions are not conducive to an accurate estimate, which must be by difference, of the change in composition of the carcass of experimental animals by the slaughter method. Hence, it seems fair to minimize the importance of this apparent exception to the rule illustrated by the remaining three comparisons. The carcass analysis data, therefore, may be considered as not out of harmony with the growth data and the nitrogen balance data in indicating a superior growth-promoting value of the 11% ration over the 8% ration.

On the other hand, the nitrogen storages secured on the 15 and 11% rations reveal no significant differences, in con-

sonance with the growth data and the nitrogen metabolism data.

The biological values of the total dietary nitrogen were computed both from the balance data (table 4) and from the slaughter data (table 3). In both cases the basal dietary nitrogen is necessarily included in the calculations, as well as the urea nitrogen and the casein nitrogen. Average values for the excretion of endogenous nitrogen in the urine (0.033 gm. per kilogram body weight) and of metabolic nitrogen in the feces (0.56 gm. per 100 gm. of dry matter consumed), obtained in the preceding experiment on the maintenance lambs, were assumed in making these calculations. The calculations from the balance data were made by the method usually employed in this laboratory (Mitchell, '24), while those made from the slaughter data were necessarily different though they possess the same significance. In the latter case, the total nitrogen storage plus the estimates of total body losses in urine and feces (endogenous + metabolic nitrogen) was divided by an estimate of absorbed nitrogen obtained by multiplying the total nitrogen intake for each lamb by the true digestibility of nitrogen computed from the results of the nitrogen balance studies.

The average biological values for the nitrogen of the basal ration, at a level of 5 to 6%, were 81 from the balance data (four cases) and 100 from the slaughter data on one lamb. For the rations containing 8% protein equivalent (34% of the nitrogen in the form of urea), the average biological values were 75 from the balance data and 74 from the slaughter data. For the rations containing 11% protein equivalent (50% of the nitrogen derived from urea), the average biological values were 63 from the balance data and 58 from the slaughter data. For the rations containing 15% protein equivalent (62% of the nitrogen derived from urea), the average biological values were 47, as obtained from the balance data, and 41, as obtained from the slaughter data. The latter values may be compared with an average biological value of 59 for the 15% casein ration obtained from the balance data on lamb 2850.

In accordance with general experience, the biological values of the urea rations decrease as the concentration of conventional protein increases, from 74 at the 8% level, to 60 at the 11% level, and to 44 at the 15% level. The values obtained from the two sets of data agree remarkably well considering that the balance data were obtained over periods of 10 days and the slaughter data over periods of 71 to 111 days.

For urea alone, Fingerling and associates ('37) have computed the efficiency of utilization on two growing calves to be 50 and 61%, using nitrogen balance data on a low-protein ration and on the same ration plus a urea supplement. The latter ration did not support maximum retention of nitrogen. These values are comparable with those obtained in this experiment for the total nitrogen of the 11% ration.

The average standard deviations of the biological values about their respective group means were 5.0, for those derived from the balance data, and 3.2 for those derived from the slaughter data. Mitchell, Burroughs and Beadles ('36) have reported an average standard deviation of 3.7 for an extensive series of biological values, each of which was secured with a group of nine or ten rats.

The 15, 11 and 8% protein equivalent rations contained, respectively, 3.16, 1.88, and 0.93% of urea on the dry basis on the average. In order to detect evidence of possible renal damage, the kidneys of all slaughtered lambs were weighed and examined histologically by Dr. C. C. Morrill of the Division of Animal Pathology and Hygiene. In the four possible comparisons of the 15 and 11% rations, the kidney weight was consistently greater on the higher level of urea, averaging for both kidneys, 92.5 gm. for the 15% lambs and 80.0 gm. for the 11% lambs. However, between the lambs on the 11 and the 8% rations no consistent difference in kidney weight was observed, the average weights being 68.8 and 72.5 gm., respectively.

The histological examination revealed no changes in structure that are not often seen in animals receiving normal

rations. Although the urine volumes on all levels of urea were measured in a few cases, no evidence of diuresis was obtained. It seems fair to conclude that, in the concentrations fed and under the conditions of these experiments, urea did not exert an appreciable toxic effect.

CONCLUSIONS

1. The addition of urea to a low-nitrogen ration that is in itself unable to support appreciable growth in lambs or even consistently to maintain nitrogen equilibrium, converts it into a ration capable of promoting a normal or nearly normal rate of growth. Such a ration need contain no more than 11% of conventional protein (N $\times$ 6.25), in which urea provides 50% of the nitrogen.

2. The biological value of the nitrogen of a ration of silage and a carbohydrate supplement, containing 5.35% of conventional protein on the dry basis, is about 82. With successive additions of urea to produce rations containing approximately 8, 11 and 15% of protein equivalent, the average biological values are 74, 60 and 44, respectively.

3. Rations of the type used in this experiment, containing up to 3.16% urea on the dry basis, do not exert any observable toxic effect on lambs over a feeding period of 110 days. At the highest level, some renal hypertrophy results, but there is no histological evidence of kidney damage.

The authors wish to acknowledge the invaluable advice and assistance of Dr. W. G. Kammlade and Mr. W. J. Hampton of the Sheep Husbandry Division in the feeding and care of the lambs employed in these experiments.

LITERATURE CITED

FINGERLING, G., B. HIENTZSCH, H. KUNZE AND K. REIFGERST 1937 Ersatz des Nahrungseiweisses durch Harnstoff beim wachsenden Rinde. Landw. Vers.-Stat., vol. 128, pp. 221–235.

MITCHELL, H. H. 1924 A method of determining the biological value of protein. J. Biol. Chem., vol. 58, pp. 873–903.

MITCHELL, H. H., E. W. BURROUGHS AND J. R. BEADLES 1936 The significance and accuracy of biological values of proteins computed from nitrogen metabolism data. J. Nutrition, vol. 11, pp. 257–274.

MITCHELL, H. H., AND T. S. HAMILTON 1931–1932 Current feeding standards for lambs can be improved. 45th Ann. Rpt. Ill. Agr. Exp. Sta., pp. 95–97.

———— 1935–1936 Lambs require less protein than commonly supposed. 49th Ann. Rpt. Ill. Agr. Exp. Sta., pp. 104–106.

STUDENT 1908 The probable error of a mean. Biometrika, vol. 6, pp. 1–25.

THE EFFECT OF HEAT ON THE AVAILABILITY OF THE IRON OF BEEF MUSCLE

HELEN G. OLDHAM
Department of Pediatrics, The University of Chicago, Illinois

TWO FIGURES

(Received for publication January 31, 1941)

In a previous paper (Oldham, Schlutz and Morse, '37) we reported that, as judged by the amount of iron retained, a normal infant was able to utilize the iron of cooked beef muscle equally as well as that of an iron salt. This finding was surprising, since Sherman, Elvehjem and Hart ('34) had reported that less hemoglobin was formed in anemic rats fed beef muscle than in those fed the same amount of iron in the form of iron salts.

In the present study we have attempted to find an explanation for the difference between our results and those of the Wisconsin group by comparing the hemoglobin levels of anemic rats after feeding equal amounts of iron as ferric chloride and as beef muscle. The beef muscle was fed both before and after heating in order to determine whether or not the process of heating affected the utilization of the iron of the meat. The study was divided into three parts in each of which matched pairs of animals received equivalent amounts of iron in different forms. In the first part ferric chloride and oven-dried meat were fed; in the second part, ferric chloride and vacuum-dried meat; in the third, oven-dried meat and vacuum-dried meat.

EXPERIMENTAL

Treatment of animals

Young male rats from mothers who had received our stock ration were weaned at 18 days of age. They were then housed in glass cages as described elsewhere (Oldham and Schlutz, '40) and fed a diet of fresh whole milk. Approximately 6 weeks were required for depletion of iron reserves and during the last 2 weeks of this period copper and manganese salts were added to the milk diet to insure complete depletion. When the hemoglobin levels were reduced to 3.0–4.0 gm. per 100 ml. of blood, the animals were paired as to litter, hemoglobin level and weight. Supplementary feedings were then begun and were continued through a period of 6 weeks.

Diets

The animals received a basal diet of milk and copper and manganese salts in addition to their supplementary feedings of either ferric chloride or meat. Fresh whole unpasteurized milk was obtained from a local dairy. Amounts fed daily were measured by pipettes into the feeding cups. A solution of copper sulphate and manganese chloride equivalent to 0.05 mg. of copper and 0.04 mg. of manganese was added to each milk cup before feeding. The supplements of ferric chloride were prepared in the laboratory from iron wire. They were measured by pipette and mixed with a small amount of milk. All supplements were given in the morning and were entirely consumed before additional food was offered. The meat was purchased as round steak, ground and dried either in an oven at 80°C. or under vacuum at room temperature. After it was dried, it was ground again in a mortar and the daily portions were weighed on an analytical balance.

In the first experiment, one group of animals received daily 68 ml. of milk and 2.0 ml. of ferric chloride ($\simeq$ 0.196 mg. Fe). Their litter mates received 40 ml. of milk, 2.57 gm. of oven-dried meat and 1 gm. of cane sugar. The cane sugar was sub-

stituted for its caloric equivalent of milk to reduce the protein content of the diet and thus make it more comparable to that of the animals receiving ferric chloride supplements. The milk in the diet of both groups of animals was increased gradually as the experiment progressed, by amounts which insured good gains. The amount of milk given was always such that it was completely consumed by all animals. It was considered essential to keep the diets isocaloric in order that the weight gains of the two groups of animals would be comparable, since the hemoglobin level on a given iron intake is affected by the total blood volume and this in turn is proportional to the body weight.

The animals in experiments 2 and 3 were treated similarly to those in experiment 1 with the exception of the supplements fed. In experiment 2 one group of animals received ferric chloride, their litter mates, vacuum-dried meat. In experiment 3 one group was fed oven-dried meat, the other vacuum-dried meat.

Methods of analysis

Samples of milk were analyzed for iron and the results obtained were used throughout the study. Each lot of meat was analyzed separately and an amount fed which kept the daily iron intake constant. The ferric chloride solutions were also analyzed for iron. The amyl alcohol thiocyanate method was used for all iron determinations.

Hemoglobin determinations were made at the beginning of each experiment and at the end of the second, fourth, fifth and sixth weeks. The method used was that of Evelyn ('36).

DISCUSSION OF RESULTS

The daily iron intakes and the average gain in hemoglobin of animals on the different supplements are shown in table 1. The average hemoglobin levels on the various supplements are also shown in figure 1. When the chief source of iron was oven-dried meat, the average hemoglobin gain was slightly,

TABLE 1

Daily iron intake and hemoglobin formation of animals receiving different supplements

EXP.	NO. OF ANIMALS	SUPPLEMENT TO MILK DIET	DAILY INTAKE OF Fe	AV. Hb. LEVEL AT BEGINNING	AV. Hb. LEVEL AT END	AV. GAIN IN Hb.
			mg.	*gm./100 cc.*	*gm./100 cc.*	*gm./100 cc.*
1	6	Oven-dried meat	0.257	3.35(2.98–3.75)	12.39(10.14–14.30)	9.04(6.59–11.16)
	6	Fe Cl_3	0.257	3.26(2.88–3.70)	11.12(10.06–13.18)	7.86(6.03– 9.48)
2	5	Vacuum-dried meat	0.260	3.68(2.98–4.07)	9.47(8.88–10.22)	5.79(5.54– 6.26)
	4	Fe Cl_3	0.261	3.60(2.98–4.02)	13.17(12.17–13.72)	9.57(9.19–10.12)
3	5	Oven-dried meat	0.264	3.56(3.14–3.86)	13.26(12.09–14.30)	9.70(8.70–10.44)
	4	Vacuum-dried meat	0.259	3.54(2.78–4.07)	7.68(7.45– 7.85)	4.14(3.76– 4.67)
Averages:		Oven-dried meat	0.260	3.45	12.79	9.34
		Fe Cl_3	0.259	3.40	12.18	8.78
		Vacuum-dried meat	0.260	3.62	8.67	4.61

but not significantly greater than that found when an equal amount of iron was fed as ferric chloride (9.3 gm. and 8.8 gm. respectively per 100 ml. of blood). When, however, the chief source of iron was meat which had not been heated, the average hemoglobin level was significantly less than that for the other two groups, even though the total daily iron intake was the same for all three groups.

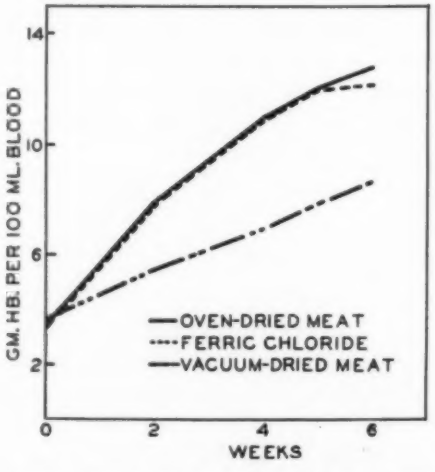


Fig. 1 Average hemoglobin formation on different supplements.

The same results are observed when individual hemoglobin values of paired animals are compared. It is evident (fig. 2) that, in each pair of animals, although slight variations occurred, the one which received oven-dried meat or ferric chloride was able to build almost twice as much hemoglobin as the litter mate who received vacuum-dried meat. It may also be noted that when one of a pair received oven-dried

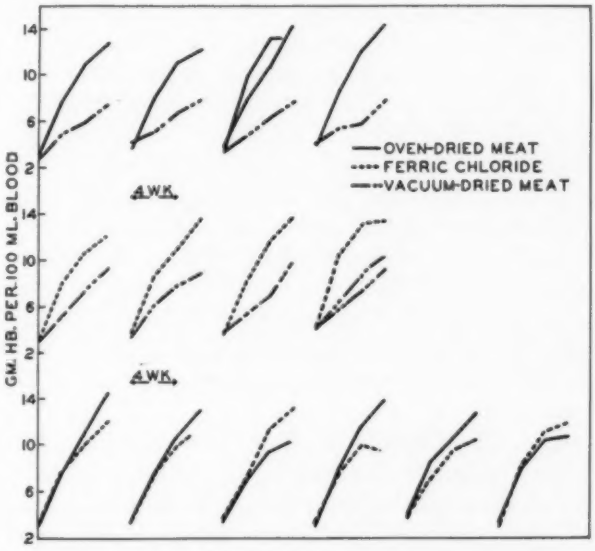


Fig. 2 Hemoglobin formation of individual animals.

meat and the other ferric chloride, the hemoglobin gains were, in every case, almost identical.

There seems to be little doubt but that at least 50% of the iron of beef is in the organic form, and is incorporated in the hemoglobin molecule. In a compilation of results from various laboratories McCance ('39) states that values ranging from 50 to 85% have been reported as representing the proportion of iron which did not react with dipyridyl reagent. The findings of Shenk, Hall and King ('34) substantiate this amount.

After analyzing the ribeye muscle of beef for muscle hemoglobin and blood hemoglobin they reported values of 0.332 to 0.454% (for the former) and 0.015 to 0.041% (for the latter).

The amount of dried meat given daily in the present study was 2.5 gm. which represented approximately 9 gm. of fresh meat. This amount of meat would contain 31 to 46 mg. of hemoglobin, if it is assumed that the percentages of muscle hemoglobin and blood hemoglobin in the meat used in this study were the same as those reported by Shenk and his co-workers. This would mean that 0.11 to 0.16 mg. of the iron intake of the animals receiving meat supplements was organic (of hemoglobin origin) since the iron content of both forms of hemoglobin according to Millikan ('39) is the same (0.345%). As the total iron content of the beef fed was approximately 0.24 mg., and according to the above calculations 0.11 to 0.16 mg. was organic, it would seem justifiable to consider at least 50% of the iron of beef to be in the organic form.

It seems therefore, that the effect of the heat was to render the organic iron of meat as available as that of an inorganic iron salt. In light of this observation, the results of our previous study seem reasonable, since the infant was fed meat which had been cooked.

The manner in which heat affects this change in the availability of iron is an open question. That heat renders the beef more digestible seems improbable, since the animals receiving the two types of meat made comparable weight gains. A possible explanation might be that heat so affects the hemoglobin molecule as to make it more easily broken down and thus more readily assimilated by the body.

SUMMARY

Young rats made anemic by a milk diet were given supplements of ferric chloride, oven-dried meat and vacuum-dried meat.

When equal amounts of iron were fed in the form of the above-named supplements approximately the same amount of hemoglobin formation was observed when the supplement was

oven-dried meat as when it was ferric chloride. Significantly less hemoglobin formation occurred when vacuum-dried meat was fed.

Heat renders the iron of beef muscle of which at least 50% is in the organic form, as available for hemoglobin synthesis as the iron of an inorganic iron salt.

LITERATURE CITED

EVELYN, K. A. 1936 A stabilized photoelectric colorimeter with light filters. J. Biol. Chem., vol. 115, p. 63.

MCCANCE, R. A. 1939 The ionizable and available iron in food. Chem. and Industry, vol. 58, p. 528.

MILLIKAN, G. A. 1939 Muscle Hemoglobin. Physiol. Rev., vol. 19, p. 503.

OLDHAM, H., F. W. SCHLUTZ AND M. MORSE 1937 Utilization of organic and inorganic iron by the normal infant. Am. J. Dis. Child., vol. 54, p. 252.

OLDHAM, H., AND F. W. SCHLUTZ 1940 The effect of different levels of vitamin B_1 and iron on the retention of iron and the fat content of normal young rats. J. Nutrition, vol. 19, p. 569.

SHENK, J. H., J. L. HALL AND H. H. KING 1934 Spectrophotometric characteristics of hemoglobins. J. Biol. Chem., vol. 105, p. 741.

SHERMAN, W. C., C. A. ELVEHJEM AND E. B. HART 1934 Further studies on the availability of iron in biological materials. J. Biol. Chem., vol. 107, p. 383.

CARBOHYDRATE STORAGE AND MOBILIZATION IN THE RAT

M. MASON GUEST [1]

Department of Physiology, College of Physicians and Surgeons, Columbia University

FIVE FIGURES

(Received for publication February 25, 1941)

I. GENERAL STANDARDIZATION OF BIOLOGICAL AND TECHNICAL PROCEDURES IN GLYCOGEN DETERMINATIONS

A striking feature of the published data on liver and muscle glycogen is the variability in concentration found among animals which were presumably under the same experimental conditions. As a result numerous animals must be used before safe conclusions can be drawn, even though the experimental changes are large. Thus, it is all but impossible to draw conclusions when the effect of the experimental procedure falls within the range of the usual control fluctuation, even with exceptionally large samples.

The results of a number of recent investigators, summarized in table 1, emphasize the variability in reported glycogen concentrations. Not only are wide deviations encountered from laboratory to laboratory, but in many cases in the results of a given author.

It appeared to be essential, therefore, to increase the biological, i.e., animal to animal, precision of the determination before entering upon a research in which the expected glycogen changes were of ordinary physiological magnitude. As a measure of the difference between animals the coefficient of dispersion, calculated from ε, has been used $\epsilon = \sqrt{\frac{\Sigma d^2}{N-1}}$. The

[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in the Faculty of Pure Science, Department of Physiology, Columbia.

TABLE 1

Liver and muscle glycogen-control values.

INVESTIGATOR	NO. OF RATS	AGE OR WEIGHT	PREVIOUS DIET	TERMINAL PROCEDURES	LIVER		MUSCLE	
					Mean	C.D.	Mean	C.D.
		24-hour fasted rats						
		days (d.) grams (g.)						
Barbour et al., '27	24 ♂, ♀	110–136 g.	Balanced	A	0.16	37.5	0.30	23.3
Blatherwick et al., '35	15 ♂	70–150 d.	Balanced	B	0.56	89.0	0.66	120.0
Blatherwick et al., '35	14 ♀	70–150 d.	Balanced	B	0.21	219.0	0.61	34.4
Buell et al., '36	9 ♂	100–150 g.	Balanced	B	0.34	....	0.46	....
Catron and Lewis, '29	16 –	100–150 g.	Balanced	C	0.09	54.4	....	....
Cori and Cori, '28 a, b	4 ♂	123–141 g.	Balanced	B	0.17	29.4	....	....
Cori, '32	15 ♂	.....	.....	B	....	....	0.53	10.0
Deuel et al., '34	10 ♂	"Adult"	Glucose	B	1.54	....	0.39	....
Deuel et al., '34	10 ♀	"Adult"	Glucose	B	0.93	....	0.40	....
Evans, '34	13 ♂, ♀	150–200 g.	Balanced	D	0.19	73.0 [1]	0.52	7.7 [1]
Hynd and Rotter, '30	....	63– 84 d.	Balanced	E	0.9	....	0.51	....
Hynd and Rotter, '30	....	63– 84 d.	Cheese	E	0.68	....	0.29	....
Karczag et al., '25	24 ♂, ♀	126–275 g.	Balanced	A	0.16	56.2	0.27	11.1
Sahyun et al., '34	9 ♂	165–235 d.	77.5% CHO	D	....	....	0.45	8.9
Sahyun et al., '34	10 ♂	165–235 d.	54.0% CHO	D	....	....	0.28	29.0
		Fed rats						
Barbour et al., '27	7 ♂, ♀	159–400 g.	Balanced	A	2.10	19.5	0.34	14.7
Britton and Silvette, '32	6 ♂, ♀	30– 60 g.	Balanced	—	1.05	16.2	0.47	10.6
Collip et al., '36	11 ♂, ♀	60– 90 d.	Balanced	B	....	....	0.53	15.1
Cori and Cori, '33	5 –	.....	.....	B		....	0.64	15.0
Deuel et al., '34	10 ♂	"Adult"	High CHO	B	3.77	....	0.43	....
Deuel et al., '34	10 ♀	"Adult"	High CHO	B	3.74	....	0.40	....
Deuel et al., '37	10 ♂	73– 76 d.	Balanced	B	4.25	14.4	....	....
Deuel et al., '37	10 ♀	73– 76 d.	Balanced	B	2.59	27.6	....	....
Evans, '34	6 –		Balanced	D	2.08	33.3 [1]	0.64	5.7 [1]
Greisheimer and Johnson, '29	♂, ♀	42 d.	45% sucrose	F	3.01	....	0.31	....
Greisheimer and Johnson, '30 b	30 ♂, ♀	40– 44 d.	Balanced	F	3.34	26.5 [2]	0.30	21.4 [2]
Greisheimer and Johnson, '30 b	33 ♂, ♀	40– 44 d.	60% starch	F	3.74	24.8 [2]	0.31	27.2 [2]
Greisheimer and Johnson, '30 b	30 ♂, ♀	40– 44 d.	60% sucrose	F	4.43	21.8 [2]	0.29	27.7 [2]
Greisheimer and Johnson, '30 b	30 ♂, ♀	40– 44 d.	60% lard	F	3.85	20.9 [2]	0.31	26.0 [2]
Greisheimer and Johnson, '30 b	30 ♂, ♀	40– 44 d.	60% casein	F	2.68	31.5 [2]	0.33	24.4 [2]
Hynd and Rotter, '30	— ♂, ♀	63– 84 d.	Balanced	E	3.1	....	0.59	....
Hynd and Rotter, '30	— ♂, ♀	63– 84 d.	Cheese	E	1.85	....	0.56	....

Terminal procedures—A = decapitation, B = amytal, C = chloroform, D = nembutal, E = stunned and decapitated, F = stunned and spinal section. C.D. = coefficient of dispersion.

[1] The deviations given were assumed to be the deviation of the mean (ϵ_M).

[2] The deviations given were assumed to be the probable error of the mean.

deviation usually given, ε_M, is a measure of the precision of the mean (Scott, '27). Since the present interest is in the precision of individual values this has been calculated to ε when necessary in the preceding summary.

Although no absolute measure of accuracy is available, larger glycogen recoveries are probably an indication that the analysis has more closely approximated the actual tissue concentration. On the basis of this criterion an improvement in accuracy also has been accomplished.

The final procedure is actually a synthesis and coordination of the published contributions of many individual workers besides ourselves.

Methods

Considerable care has been exercised in the selection and preparation of the subjects. The reported results include only determinations on 100-day-old, male albino rats of the Sherman strain.

The Rockland dry pellet rat diet [2] (which contains 50% carbohydrate, 25% protein and 5% fat) has been used. All fed rats have been subjected to a 48-hour inanition period preceding the final 12 hours of feeding. This insures that the animals eat uniformly at the selected time. To simulate the natural nocturnal feeding conditions the animal room was lighted until the food was provided and was kept dark until the last animal had been removed for sampling.

The amount of food that had passed the stomach during the final feeding period was determined in the following manner. The rats, in individual cages, were allowed food ad libitum during the allotted period. After removal of the tissues for analysis, the stomach contents were dried over a steam bath and the weight deducted. Figure 1 indicates the relation between the amount of food per gram of body weight which passed the stomach and the liver glycogen concentration. By means of such a curve it is possible to adjust all liver glycogen

[2] Manufactured by the Aready Farms Milling Company, Chicago, Ill.

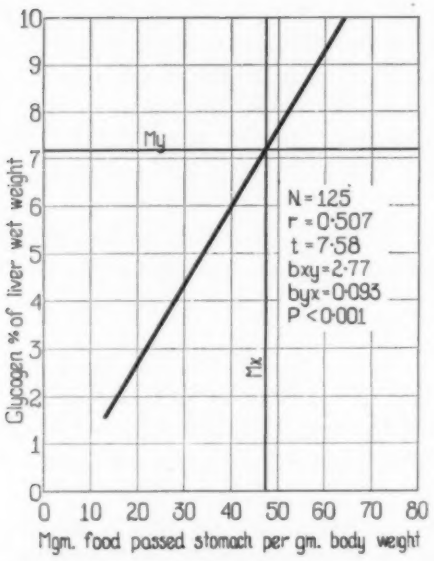


Fig. 1 Relationship between the food which passed the stomach and the percentage of liver glycogen. The heavy r line corresponds to the least square criterion for the plot of the individual points. The regression lines are not shown, but may be plotted from the constants given.

Explanation of symbols:

N = the number of experimental animals.

M_x and M_y are the means of the x and y values respectively.

r, the correlation coefficient $= \Sigma xy / \sqrt{\Sigma x^2 \times \Sigma y^2}$. This may take any value between -1 and $+1$. If r is zero the two factors are independent, while the nearer r approaches ± 1, the greater the degree of correlation. The deviation of r $\left(\sqrt{\frac{1 - r^2}{N - 1}}\right)$ is significant only for large samples (>100) and with a normal distribution of r.

t = test of significance of the correlation coefficient in terms of the standard error. Given t and N, the probability (P) may be obtained from Fisher's table.

The regression equations may be expressed as $X = M_x + b_{xy}\ (y - M_y)$ and $Y = M_y + b_{yx}\ (X - M_x)$ where X is the average value of x for a given value of y, M_x is the mean of the x-values and b_{xy} is the regression coefficient of x on y.

concentrations to a standard amount of food entering the intestine. It was found, however, that in the reported series the elimination of all animals having had less than 40 mg. of food per gram of body weight passed the stomach during the feeding period gave essentially the same glycogen precision as by adjustment. On this basis less than 5% of all animals have been rejected.

The effects of small temperature fluctuations are uncertain, but rats kept at 12°C. for 12 to 14 hours prior to sampling had 45% less liver glycogen and 12% less muscle glycogen than similar animals at 28°. We therefore, like Barbour et al. ('27), maintained the animals at $28 \pm 1°$ during the preliminary procedure.

Nembutal has been used as the anesthetic. The advantages of a short induction period are obvious and the high glycogen concentrations, "normal" blood sugar and lactic acid [3] concentrations which we have obtained are indicative that glycogenolysis with this anesthetic has been minimal.

The technic of administration of the anesthetic has been found to be important. Less excitation occurred if the rat's head was covered with a small towel while still in its cage, and kept covered until after injection. With 80 mg. of nembutal per kilogram of body weight, intraperitoneally, the animal passes into a state of deep anesthesia within 2 to 3 minutes.

The gastrocnemius muscles have been frozen in situ. We have been unable to show any significant difference between the two gastrocnemii of the same animal when both are frozen in situ, yet in ten experiments when one was so frozen and the other muscle carefully dissected out and then frozen immediately, the glycogen concentration was significantly lower (0.87 ± 0.02) than that of the contralateral muscle (1.09 ± 0.02).

[3] Although lactic acid determinations were not carried out on all animals, the results obtained indicate that a minimal change has occurred during the terminal procedures. Thus a group of twelve fed rats gave the following mean values: muscle glycogen, $0.93 \pm 0.02\%$; blood sugar, 104 ± 3 mg. per cent and lactic acid, 19.5 ± 1.6 mg. per cent.

In addition, maintenance of the tissue in the frozen state prior to the action of the alkali retards enzymatic action.

In liver and muscle glycogen determinations any extraneous tissue constituent tends to decrease both the accuracy and precision of measurement (Guest, '38). Fenn ('39) has recently shown that the blood content of the liver may be reduced as much as 5% by a severe hemorrhage prior to its removal. Consequently 4 to 5 cc. of blood were drawn as rapidly as possible by heart puncture; the abdomen was quickly opened by longitudinal incision, and the liver was immediately removed and packed in CO_2 snow. The subcutaneous fat which might be included in the muscle sample was quickly stripped away after the leg had been skinned prior to being chilled.

The frozen tissue was crushed to a powder by the method described by Graeser et al. ('34). The crushed tissues are placed on waxed paper alternately with CO_2 snow. In this way a number of tissue samples may be crushed in sequence and preserved frozen for mixing and introduction into the KOH. The connective tissue was separated from the muscle by shifting the powdered tissue back and forth a few times on the waxed paper. The tissue was weighed in KOH just above the dew point. This avoids loss by evaporation and gives no indication of glycogen loss.

The Blatherwick et al. ('35) modification of the Good, Kramer and Somogyi method ('33) has been used, further modified as noted. After the acid hydrolysis, glucose was determined by the method of Shaffer and Hartmann ('21), with use of the table and the precautions given by Duggan and Scott ('26). The reducing action of non-glycogen substances does not seem to be significantly great. This was shown by adding the supernatant fluid from centrifuged saliva to the crushed tissue and incubation at 40°C. for 30 minutes in a pH 6.9 buffered solution. Less than 0.05% reducing substance per gram of wet tissue was recovered when the digested tissue was carried through the glycogen method.

Results

In a series of sixty-seven rats, standardized as described, a mean liver glycogen (as glucose) of 8.25 ± 0.79 gm. per 100 gm. of wet tissue and a mean muscle glycogen of 1.06 ± 0.13 gm. per 100 gm. of wet tissue were obtained. The coefficients of dispersion were 9.6 and 12.3 respectively.

When these results are compared with those obtained by other workers (see table 1) the increase in the level and the decrease in the coefficients of dispersion are apparent. In another series of twenty rats in which (1) the temperature, (2) the light, (3) the food intake, (4) excitation and (5) the hemorrhage were uncontrolled the mean glycogen values were respectively 5.6 ± 1.2 and 0.81 ± 0.33 for liver and muscle. The differences in both recovery and precision between the two groups are obvious.

II. NUTRITIONAL CONTROL AS A MEANS FOR THE STANDARDIZATION OF LIVER AND MUSCLE GLYCOGEN CONCENTRATIONS AT DESIRED LEVELS

The possibility of experimental modification in tissue glycogen concentrations is frequently dependent on the control levels. Controlled variation of the nutritional state appears to be the most physiological and direct means of establishing these control concentrations at a convenient level. Procedures are described which have been found capable of varying the liver glycogen concentration from practically zero to over 8% of the tissue wet weight. In this work certain additional facts were brought out which are of interest.

Methods

In addition to the Rockland rat diet (designated "carbohydrate"), two others have been used: One, a protein diet, consisting of meat scraps,[4] contained 55% protein, 5.6% fat, and less than 1% carbohydrate. The other, designed to promote gut activity while furnishing little or no caloric value,

[4] Armour's.

contained agar-agar (100 gm.), mineral oil (4 gm.) and Liebig's extract (1 gm.); these ingredients were thoroughly mixed by grinding.

All animals were fed the Rockland rat diet until 7 days before the tissue analysis, and some until the preliminary fasting periods. During the 7 days preceding sampling the protein diet animals were allowed to eat the meat scraps ad libitum except during the preparatory fasting periods. Animals fed on both diets were fasted for 48 hours as a preliminary to the final 12-hour feeding. The agar-fed animals received the Rockland rat diet followed by 24 hours of agar-agar feeding. In each experimental group carbohydrate and protein pre-fed rats were fasted at atmospheric pressure and served as controls for animals in the same nutritional states which were fasted at one-half atmosphere pressure as described by Evans ('34).

Results

The mean values are summarized in table 2. The total blood sugar and liver and muscle glycogen are shown in figure 2. In this figure the carbohydrate-fed group is considered as 100%, and the values for each of the other groups are given in relation to this.

Discussion

By means of the feeding procedures that have been described, it is possible to obtain liver glycogen concentrations of less than 0.1% to over 8% of the liver wet weight. The muscle glycogen concentrations exhibit less variation between the groups, the lowest being 0.60% for the agar-fed rats and the highest 1.06% for the carbohydrate-fed group.

The effect of the proportion of protein upon the glycogen concentrations of the tissues is dependent upon whether the analysis immediately follows feeding or 24 hours of inanition. Carbohydrate-fed rats yielded over three times as much liver glycogen and a significantly greater muscle glycogen con

centration than those similarly fed the protein diet. Our results thus confirm the conclusions of Greisheimer and Johnson ('30 a) relative to liver glycogen and give evidence of a related muscle glycogen behavior.

In contrast to those fully fed, animals pre-fed with meat scraps and fasted for 24 hours invariably exhibited a higher liver glycogen concentration than those pre-fed with the carbohydrate diet. The probability of this difference is high when both groups are maintained at atmospheric pressure.

TABLE 2

The tissue glycogen and blood sugar in various nutritional conditions.

GROUP NO.	DIET	INANITION	NO. OF RATS	MEAN LIVER GLYCOGEN AND PRECISION OF MEAN	$\sqrt{\frac{\Sigma d^2}{N-1}}$	MEAN MUSCLE GLYCOGEN AND PRECISION OF MEAN	$\sqrt{\frac{\Sigma d^2}{N-1}}$	MEAN BLOOD SUGAR AND PRECISION OF MEAN	$\sqrt{\frac{\Sigma d^2}{N-1}}$
1	CHO	None	67	8.25±0.10	±0.79	1.06±0.02	±0.13	104±2	±15
2	Prot.	None	8	2.52±0.18	±0.52	0.81±0.02	±0.06	105±4	±12
3	CHO	24 hr.	10	0.35±0.09	±0.30	0.68±0.02	±0.06	91±6	±18
4	Prot.	24 hr.	10	1.01±0.07	±0.23	0.70±0.02	±0.06	110±3	±11
5[1]	CHO	24 hr.	10	1.77±0.18	±0.56	0.85±0.04	±0.12	123±4	±13
6[1]	Prot.	24 hr.	10	3.74±0.25	±0.79	0.96±0.05	±0.17	129±4	±13
7	Agar	A	13	0.08±0.01	±0.05	0.60±0.01	±0.05	104±2	± 9
8	CHO	B	8	6.24±0.20	±0.55			102±2	± 7
9	CHO	C	5	5.91±0.22	±0.48			103±2	± 4

[1] Maintained at one-half atmosphere during the inanition period.

A = Agar-agar fed during the final 24-hour period.

B = Allowed 5.1 gm. Rockland rat diet per 100 gm. rat during the final 12-hour feeding period.

C = Allowed 4.0 gm. Rockland rat diet per 100 gm. rat during the final 12-hour feeding period.

Glycogen given as glucose in per cent of tissue wet weight.

Blood sugar given in milligrams per cent.

The same relation, but with greater difference, is found when the rats are subjected to a pressure of one-half atmosphere during the fasting period. Differences in muscle glycogen and blood sugar between the protein and carbohydrate groups are not statistically significant, but seem to parallel that of the liver glycogen.

These results are not in agreement with the conclusions of most other workers. MacKay and Bergman ('33), working with three rats per group, reported a higher glycogen concentration in the livers of carbohydrate pre-fed animals after 24 hours of starvation than in those pre-fed on a diet predominately protein or fat. However, after 48 hours of fasting their rats which were pre-fed with the high protein diet showed higher liver glycogen than did those pre-fed with a high carbohydrate diet. Bollman and Mann ('36) also reported similar results with dogs, but did not state the number of animals used nor the experimental deviations. The workers cited fed their experimental diets from 18 to 45 days. Markedly unbalanced diets, when fed over a long period of time, may conceivably alter the liver function.

Our results thus indicate that an adequate supply of carbohydrate maintains the liver glycogen at a higher level during feeding than a diet deficient in carbohydrate, but high in protein. In contrast, 24-hour fasted animals are better able

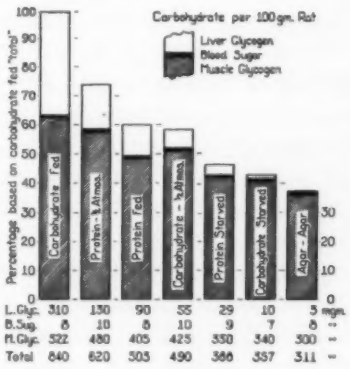
Fig. 2 Representation of the distribution of liver and muscle glycogen and blood sugar in the experimental groups. The calculation of the total liver glycogen is based on the weight of the liver and the animal and the determined glycogen percentage. The muscle glycogen value is based on the assumption that the muscle tissue makes up 50% of the body weight and that the flexor leg muscles are representative of the muscles as a whole. The total sugar in the blood is calculated from the blood sugar determination and the assumption that the blood constitutes 8% of the body weight.

Fig. 3 The relation between the blood sugar and the log. value of the liver glycogen concentration. In all fed groups studied the mean blood sugar is approximately 104 mg. per cent, regardless of the liver glycogen concentration. The r line for the fasted groups and the locations of the means are indicated. For the forty fasted animals, $r = 0.770$, $t = 6.67$, $P = 0.001$, $b_{xy} = 0.002$ and $b_{yx} = 29.16$. The results of a few other investigators are also shown. Because of lack of space on the graph the reference Cori, '28 a, '28 b, refers to Cori and Cori, '28 a, '28 b; MacKay, '33, to MacKay and Bergman, '33, and Lawrence, '31, to Lawrence and McCance, '31.

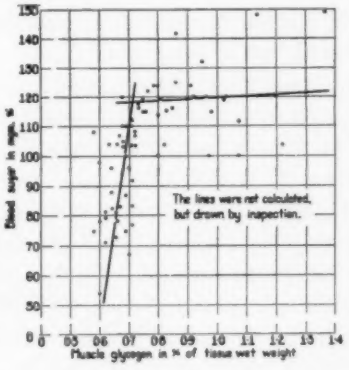
Fig. 4 Graphical representation of the relation between the muscle glycogen and blood sugar concentrations in fasted animals.

Fig. 5 The relation between muscle and liver glycogen in fasted and in fed animals. The upper curves have been recalculated on a total liver and muscle glycogen basis as explained in the text. The lower curves give the relations between the concentrations.

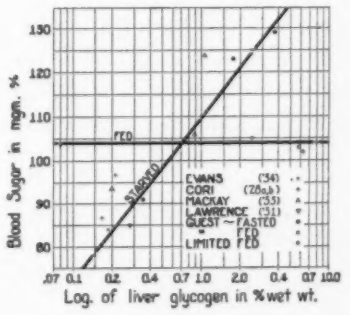
to maintain the liver glycogen when pre-fed a high protein diet. For protection during periods of inanition, a high protein diet for a comparatively short period previous to the fast, is indicated.


Carbohydrate per 100 gm. Rat
Liver Glycogen
Blood Sugar
Muscle Glycogen
Percentage based on carbohydrate fed "total"
Carbohydrate Fed
Protein + ½ Atmos.
Protein Fed
Carbohydrate + ½ Atmos.
Protein Starved
Carbohydrate Starved
Agar - Agar
L.Glyc. 310 130 90 55 29 10 5 mgm.
B.Sug. 8 10 8 10 9 7 8 "
M.Glyc. 522 480 405 425 330 340 300 "
Total 840 620 503 490 366 357 311 "

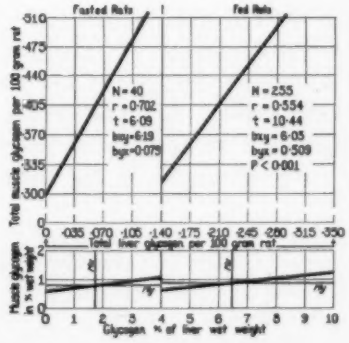
2



4



3



5

The character of the metabolic changes which occur during fasting may become more apparent through an experimental treatment which exaggerates or amplifies these changes. Evans ('34, '36) reported that when he kept rats at a pressure of one-half atmosphere during the period of fasting, the liver glycogen was sensibly the same as that of comparable fully-

fed animals, whereas the nitrogen excretion was higher. Evans's data together with our own results provide strong evidence that gluconeogenesis from protein is accelerated under the low pressure conditions and that protein is made more readily available for gluconeogenesis during periods of fasting by means of protein excess in the diet previous to inanition.

The effect of an active gut in fasted animals was investigated by means of the agar-agar diet. Although the animals on this diet were without absorbable food for the same length of time as were the 24-hour fasted animals, both the liver and muscle glycogen concentrations were found to be significantly lower than with any of the fasted groups. It is worth noting that three animals which were given magnesium sulfate by stomach tube and then fasted for 24 hours showed liver and muscle glycogen concentrations (0.15 and 0.64) comparable to the agar animals. Several interpretations, not mutually exclusive, of these low values are possible: (1) Increased gut activity may deplete the glycogen stores through increased energy demands. (2) The agar-agar acting as a mild cathartic may empty the gut of absorbable materials more effectively than fasting. (3) The active gut may call forth circulatory, hormonal or nervous responses which influence glycogenesis or glycogenolysis in the liver.

III. THE INTERRELATIONSHIPS OF THE CONCENTRATIONS OF TISSUE CARBOHYDRATES

These were studied by a regression analysis (Fisher, '36) of the data accumulated in part II and representing about 300 animals. In all cases the fasted and fed animals were treated separately.

1. Blood sugar-liver glycogen. No demonstrable relationship has been found between the blood sugar and the liver glycogen in any of the fed groups. The mean blood sugars in these groups show no significant differences, regardless of the liver glycogen concentration. In figure 3 the mean values

for all of the fed groups fall on or near a line parallel to the x-axis.

In contrast to the results in the fed groups, the mean blood sugar concentrations for the fasted groups vary directly as a function of the log. value of the liver glycogen concentration (fig. 3). The correlation coefficient (r) for the fasted individuals is 0.770 and t is 6.67. Thus the probability of an important physiological relation is indicated; the physiological significance being measured by r and the probability by t.

The conclusion that different mechanisms are responsible for the blood glucose-liver glycogen relationship in feeding and in fasting animals is not warranted by the evidence available. However, it does appear that physiological adjustments are made in the transition between the two nutritional states. It may be significant that Cori, Cori and Schmidt ('39) in phosphorylation studies obtained more potent phosphorylase extracts from the livers of well-fed rabbits than from those of fasting animals.

2. Blood sugar-muscle glycogen. Although no relationship could be found between the blood sugar and muscle glycogen concentrations of the fed animals, in the fasted rats there appears to be a definite tendency for the individual muscle glycogen-blood sugar values to fall about the curve indicated in figure 4. This type of curve is not susceptible to treatment by ordinary regression methods, as there appears to be an inflection at a muscle glycogen concentration of about 0.75% and a blood sugar concentration of about 120 mg. per cent. Thus the relationships between the blood sugar and the muscle glycogen appear to be made up of two components. Below the inflection the muscle glycogen decreases gradually while the blood sugar falls rapidly, indicating the maintenance of a minimal muscle glycogen, even though the blood sugar values are low. Above the inflection the blood sugar, although showing considerable variation, exhibits no general tendency toward higher concentrations as the muscle glycogen increases to 1.40%. This appears to indicate that the muscles are capable of acting as carbohydrate reservoirs when the blood sugar

concentration rises above a physiological level. It is evident that such a mechanism would be effective in conserving carbohydrate and in preventing the blood sugar from reaching the renal threshold.

3. Liver-muscle glycogen. The correlation between the liver and muscle glycogen concentrations for both the fed and the fasted rats is high. The calculated straight lines which best represent the distribution of the individuals are shown in the lower parts of figure 5.

The principal difference in the curves representing the two nutritional states is that, although they are roughly parallel, the straight-line relationship of the fasted group is displaced upward on the muscle glycogen axis. Thus a greater percentage of muscle glycogen is found in the fasted animals per unit percentage of liver glycogen than in the fed rats. This displacement may be a reflection of decreased muscular activity in the fasted rats or it may be indicative of a generally altered metabolism with redistribution of the carbohydrate stores. Both of these factors are possibly involved.

In contrast with the large differences between the extremes of liver and muscle glycogen concentrations, the total liver glycogen, when plotted against the total muscle glycogen, exhibits a more nearly one to one proportionality. The latter relationships are shown in the upper part of figure 5. In estimating the total muscle glycogen, the skeletal muscles were assumed to constitute 50% of the body weight and the glycogen percentage in the flexor leg muscles was assumed to be representative of the muscles as a whole. The total liver glycogen value is based on the actual wet weight of the liver and the determined glycogen concentration. These have been recalculated on the basis of a 100-gm. rat. Although the total muscle glycogen is about ten times that of the total liver glycogen, they tend to increase or decrease together. In the economy of the organism, therefore, the greater mass of muscle tissue appears to be a major factor in sustaining its relatively constant glycogen concentration.

SUMMARY

1. By means of the described procedures the recovery of glycogen from the liver and muscle of fed rats is higher than has been previously reported with a substantial reduction in the coefficient of dispersion.

2. (a) By means of nutritional control, liver glycogen concentrations may be established to a practical precision at any desired level (from 0 to 8% of the tissue, wet weight). (b) Rats on a diet predominating in carbohydrate have a significantly higher liver glycogen concentration at the end of the feeding period than rats on a diet high in protein. However, the reverse is true after the food has been withheld for 24 hours. (c) Maintenance of the animals at a pressure of one-half atmosphere during the fasting period exaggerates the effects of the pre-inanition diets on the liver and muscle glycogen and the blood sugar concentrations.

3. (a) It has been indicated by a regression analysis that a positive physiological relation exists between the blood sugar and the log. value of the liver glycogen concentration in fasted rats and between the liver and muscle glycogen concentrations in both fed and fasted rats. (b) Indications are given of a relationship between the blood sugar and the muscle glycogen concentrations in fasted rats.

The author wishes to express his sincere thanks and deep appreciation to Dr. E. L. Scott for his invaluable guidance and assistance; to Dr. J. J. McBride for his aid and suggestions; to Dr. W. S. Root for suggestions in the preparation of the manuscript; and to Miss Ruth A. Rawson for her technical assistance.

LITERATURE CITED

BARBOUR, A. D., I. L. CHAIKOFF, J. J. R. MACLEOD AND M. D. ORR 1927 Influence of insulin on liver and muscle glycogen in the rat under varying nutritional conditions. Am. J. Physiol., vol. 80, p. 243.

BLATHERWICK, N. R., P. J. BRADSHAW, M. E. EWING, H. W. LARSON AND S. D. SAWYER 1935 The determination of tissue carbohydrate. J. Biol. Chem., vol. 111, p. 537.

BOLLMAN, J. L., AND F. C. MANN 1936 The physiology of the impaired liver. Ergebn. d. Physiol., vol. 38, p. 445.

BRITTON, S. W., AND H. SILVETTE 1932 Effects of cortico-adrenal extract on carbohydrate metabolism in normal animals. Am. J. Physiol., vol. 100, p. 693.

BUELL, M. V., I. A. ANDERSON AND M. B. STRAUSS 1936 On carbohydrate metabolism in adrenalectomized animals. Am. J. Physiol., vol. 116, p. 274.

CATRON, L. F., AND H. B. LEWIS 1929 The formation of glycogen in the liver of the young white rat after the oral administration of glycerol. J. Biol. Chem., vol. 84, p. 553.

COLLIP, J. P., D. L. THOMSON AND G. TOBY 1936 The effect of adrenaline on muscle glycogen in adrenalectomized, thyroidectomized and hypophysectomized rats. J. Physiol., vol. 88, p. 191.

CORI, C. F., AND G. T. CORI 1928 a The fate of sugar in the animal body. VIII. The influence of insulin on the utilization of glucose, fructose and dihydroxyacetone. J. Biol. Chem., vol. 76, p. 755.

———— 1928 b The mechanism of epinephrine action. II. The influence of epinephrine and insulin on the carbohydrate metabolism of rats in the postabsorptive state. J. Biol. Chem., vol. 79, p. 321.

———— 1933 A comparison of total carbohydrate and glycogen content of mammalian muscle. J. Biol. Chem., vol. 100, p. 323.

CORI, G. T. 1932 Carbohydrate changes during anaerobiosis of mammalian muscle. J. Biol. Chem., vol. 96, p. 259.

CORI, G. T., C. F. CORI AND G. SCHMIDT 1939 The role of glucose-1-phosphate in the formation of blood sugar and synthesis of glycogen in the liver. J. Biol. Chem., vol. 129, p. 629.

DEUEL, H. J., JR., M. GULICK, C. F. GRUNEWALD AND C. H. CUTLER 1934 The sexual variation in carbohydrate metabolism. III. The comparative glycogen and fat content of the liver and muscles of rats and guinea pigs. J. Biol. Chem., vol. 104, p. 519.

DEUEL, H. J., JR., J. S. BUTTS, L. F. HALLMAN, S. MURRAY AND H. BLUNDEN 1937 The sexual variation in carbohydrate metabolism. IX. The effect of age on the sex difference in the content of liver glycogen. J. Biol. Chem., vol. 119, p. 617.

DUGGAN, W. F., AND E. L. SCOTT 1926 A critical examination of four methods commonly used for the determination of sugar in the blood. J. Biol. Chem., vol. 67, p. 287.

EVANS, G. 1934 The effect of low atmospheric pressure on the glycogen content of the rat. Am. J. Physiol., vol. 110, p. 273.

———— 1936 The adrenal cortex and endogenous carbohydrate formation. Am. J. Physiol., vol. 114, p. 297.

FENN, W. O. 1939 The deposition of potassium and phosphate with glycogen in rat livers. J. Biol. Chem., vol. 128, p. 297.

FISHER, R. A. 1936 Statistical methods for Research Workers, 6th ed., Oliver and Boyd.

GOOD, C. A., H. KRAMER AND M. SOMOGYI 1933 The determination of glycogen. J. Biol. Chem., vol. 100, p. 485.

GRAESER, J. B., J. E. GINSBERG AND T. E. FRIEDEMANN 1934 A method for the analysis of tissues. J. Biol. Chem., vol. 104, p. 149.

GREISHEIMER, E. M., AND O. H. JOHNSON 1929 Glycogen formation in rats. Am. J. Physiol., vol. 90, p. 369.

———— 1930 a Glycogen formation in rats. Am. J. Physiol., vol. 93, p. 653.

———— 1930b Glycogen formation in rats. I. Diets containing about sixty per cent of the total caloric value in the form of starch, sucrose, lard and casein. Am. J. Physiol., vol. 94, p. 11.

GUEST, M. M. 1938 On the determination of glycogen in muscle. J. Biol. Chem., vol. 123, p. xlviii.

HYND, A., AND D. L. ROTTER 1930 Studies on the metabolism of animals on a carbohydrate-free diet. I. The distribution of glycogen and fat in the liver of animals fed on a carbohydrate-free diet. Biochem. J., vol. 24, p. 1390.

KARCZAG, L., J. J. R. MACLEOD AND M. D. ORR 1925 The use of the albino rat in insulin standardization. Trans. Roy. Soc. Can., vol. 19, p. 57.

LAWRENCE, R. D., AND R. A. MCCANCE 1931 The effect of starvation, phloridzin, thyroid, adrenaline, insulin and pituitrin on the distribution of glycogen in the rat. Biochem. J., vol. 25, p. 570.

MACKAY, E. M., AND H. C. BERGMAN 1933 The influence of the preceding diet upon the rate of glucose absorption and glycogen synthesis. J. Nutrition, vol. 6, p. 515.

SAHYUN, M., R. SIMMONDS AND H. WORKING 1934 The effect of diet on the distribution of glycogen in the skeletal muscle of the rat. Am. J. Physiol., vol. 108, p. 708.

SCOTT, E. L. 1927 What constitutes an adequate series of physiological observations? J. Biol. Chem., vol. 73, p. 81.

SHAFFER, P. A., AND A. F. HARTMANN 1921 The iodometric determination of copper and its use in sugar analysis. II. Methods for the determination of reducing sugars in blood, urine, milk and other solutions. J. Biol. Chem., vol. 45, p. 365.